REMARKS

Applicants submitted a Submission with Request for Continued Examination on April 9, 2008 in response to the final Office Action mailed October 9, 2007. Applicants wish to thank Examiner Lankford for the personal Interview held on July 14, 2008, with the undersigned, and the helpful comments provided therein. During the interview, the undersigned and Examiner Lankford discussed possible amendments to overcome the rejection under 35 U.S.C. § 103(a), including the Katz and Peterson references, and the unexpected advantages of Applicants' claimed compositions. The Examiner agreed to consider a Supplemental Amendment. In the present Supplemental Amendment, the status identifiers are relative to the claims filed on April 9, 2008.

Upon entry of the foregoing amendments, Claims 93, 94, 96-105, 107-116, and 118-127 are pending. Applicants have cancelled Claims 95, 106, and 117 without prejudice to, or disclaimer of, the subject matter contained therein. Applicants maintain that the cancellation of a claim makes no admission as to its patentability, and reserve the right to pursue the subject matter of the cancelled claims in this or another patent application. Applicants have amended Claims 93, 96, 104, 105, 107-112, 114-116, 118-123 and 125, and have added new Claims 126 and 127. he amendments add no new matter and are fully supported by the specification and claims as originally filed. Support for the amendments can be found, for example, on page 3, lines 16-21, page 8, lines 4-14, page 12, line 12-page 13, line 10, page 14, lines 1-5, page 15, lines 11-13, page 16, lines 8-10, page 27 lines 11-26, page 31, line 13- page 33, line 2, Example 1, and elsewhere throughout the specification.

Applicants' Claimed Compositions Are Non-Obvious Under 35 U.S.C. § 103(a) In View of the Cited Art

In the final Office Action mailed October 9, 2007, the Examiner rejected Claims 93-125 under 35 U.S.C. § 103(a) as allegedly being unpatentably obvious over U.S. Patent No. 6,777,231 to Katz et al. ("Katz") or over U.S. Patent No. 6,200,606 to Peterson et al. ("Peterson"). The Examiner stated that both Katz and Peterson teach compositions comprising adipose-derived stem cells in a complex mixture, and maintained that the only difference

between Applicants' claimed compositions and the compositions taught by Katz and Peterson is the concentration of the cells and tissues contained therein. The Examiner stated that, absent evidence that the concentration of the adipose-derived stem cells is critical, the alleged differences do not render the claims patentable over the cited art. In addition to Applicants' remarks set forth in the Submission with Request for Continued Examination filed on April 9, 2008, kindly consider the following remarks.

As discussed during the Interview on July 14, 2008, the compositions recited in Claims 93, 94, 96-105, 107-116, and 118-127 of the instant application are fundamentally different from the compositions of Katz and Peterson. Applicants' claimed compositions relate to mixtures made by combining unprocessed adipose tissue, *i.e.*, intact, non-disaggregated adipose tissue, with a suspension of a concentrated population of disaggregated adipose-derived cells that comprises disaggregated adipose-derived stem cells. As amended, Applicants' claims specify that the frequency of disaggregated adipose-derived stem cells among the nucleated cells in the concentrated population of disaggregated adipose derived cells is at least 0.1%.

In contrast to Applicants' claimed mixtures, the compositions of both Katz and Peterson are derived from disaggregated adipose tissue alone, and are devoid of any unprocessed adipose tissue and intact, non-disaggregated adipose tissue fragments. Both references teach the desirability complete disaggregation and separation of stem cells from adipose tissue matrix, e.g., unprocessed adipose tissue. (See, Submission with Request for Continued Examination at 7-10). Thus, Katz and Peterson teach compositions that lead the skilled artisan *away* from mixing unprocessed adipose tissue comprising intact, non-disaggregated adipose tissue fragments with disaggregated adipose-derived cells. (See, Submission With Request for Continued Examination, at 9-10).

Numerous articles from peer-reviewed journals confirm that populations of disaggregated adipose-derived cells, *e.g.*, the cell populations described in Katz and Peterson, are not suitable for autologous fat transfer, either alone, or when the cells are seeded onto various lattices, matrices, or scaffolds. (See, Declaration of John K. Fraser, Ph.D., Submitted April 9, 2008, and Exhibits annexed thereto). As discussed during the interview, implants comprising disaggregated adipose-derived stem cells seeded on a lattice, scaffold, or matrix (such as the types mentioned in Katz and Peterson) are unsuitable because they are resorbed over time.

Furthermore, the cell-seeded scaffolds and matrices do not have the characteristics of native soft tissue, rendering them unsuitable for a large number of implants. (See, Fraser Decl., ¶¶ 5-7).

Furthermore, as discussed during the interview, Applicants' claimed mixtures are fundamentally different from fat alone, i.e., compositions that include unprocessed, non-disaggregated adipose tissue, but that do not include the suspension of concentrated disaggregated adipose-derived cell populations comprising adipose derived stem cells. Numerous studies, including Applicants' working examples, have shown that implants comprising unprocessed adipose tissue alone are not suitable for implantation, and are resorbed over time. For example, Esrek et al. attempted autologous fat transfer with unprocessed adipose tissue in more than 100 patients. Esrek et al. (1991) Plast. Reconst. Surg. 87(2):219-228, submitted herewith as **Exhibit A.** Esrek reports that "injection of 10 to 50 cc of autolgous adipose tissue into affected areas resulted in some cells (perhaps 10 percent) surviving over two years." (Esrek et al., Absstract). Ersek emphatically states "I do *not* recommend autologous fat injection as a permanent transplant at this time, but I am continuing investigations to improve survival. . .An anonymous survey of the opinions of my patients show a low regard by them for the results." (Id.) Esrek concludes that his "disappointing results, even with repeated injections lead [him] to the conculsion that very little, if any of this autologous fat survives in its new site... It appears as though [a] 10 percent survival rate may be the most one can hope for with current state of the art." (Id. at 226, Col. 1). Applicants' own experimental data confirms the retention problems associated with grafts of unprocessed adipose tissue alone. (See, e.g., Specification, Example 1, showing improved graft weight and vascularity of the claimed mixtures, compared to unprocessed adipose tissue alone). Many of the peer reviewed articles referenced in the Fraser Declaration also attest to the shortcomings of unprocessed adipose tissue as a graft.

With respect to the concentration of the adipose derived stem cells in the claimed mixture, a composition of unprocessed adipose tissue obtained by liposuction, excision, etc., will not meet the limitations of Applicants' current claims because it does not contain disaggregated adipose derived stem cells. That is, in the absence of a processing step to liberate or free the stem cells from within the adipose tissue matrix, such as, for example, a collagenase digestion step, the stem cells remain bound in matrix material and are not disaggregated as specified in the current claims. (See, Fain et al. (2004) Endocrinology 145(5):2273-2282, at 2278, Col. 2,

Submitted herewith as **Exhibit B**) (See also, Björntrop, et al. (1978) J. Lipid Res. 19:316-324, at 318, Col. 1, 3rd para, submitted herewith as **Exhibit C**).

Furthermore, the process of liposuction, excision, etc. alone, without disaggregation, will not yield a composition approaching Applicants' recited frequency of disaggregated adipose derived stem cells of at least 0.1% of the nucleated cells. Applicants note that liposuction and other adipose tissue harvesting methods may involve breakage of blood vessels and contamination of the adipose tissue with blood. However, these stem cells present in the blood would not be adipose-derived stem cells as recited in the current claims. Some studies have detected the presence of stem cells in the blood, for example. However, the frequency of the stem cells in the peripheral blood has been reported to range of 0.0002% to 0.00003% [1] to 5.6 per million cells *i.e.*, 0.00053%[2], as measured by a CFU-F assay. Thus, in addition to not being adipose-derived stem cells, the frequency of contaminating stem cells among the nucleated cells from blood is well below the frequency (*e.g.*, by at least 200-fold) of at least 0.1%, as recited in Applicants' claims.

After disaggregation, the concentration of disaggregated adipose derived stem cells may vary depending on the site of adipose tissue harvest, e.g., abdomen, waist, hip/thigh. However, studies have reported that the absolute number of nucleated cells obtained after disaggregation does not vary significantly regardless of the site of adipose tissue harvest. The authors concluded that the frequency of disaggregated adipose derived stem cells ranges from between 0.1% to 12.5% of disaggregated nucleated cells. (See, e.g., Jurgens et al. (2008) Cell Tiss. Res. 332:415-426, at 415, Col. 1 and Table 1, Submitted herewith as **Exhibit D**) The frequency of disaggregated adipose-derived stem cells in disaggregated adipose tissue from various removal sites reported in Jurgens et al. is consistent with Applicants' recited frequencies.

By combining the population of disaggregated adipose-derived cells comprising disaggregated adipose-derived stem cells with non-disaggregated adipose tissue, Applicants are able to achieve unexpected benefits over adipose-derived tissue implants known in the art, which concerned either fat alone or disaggregated adipose-derived stem cell containing populations alone or with synthetic matrices or scaffolds,. Without mixing unprocessed adipose tissue with a

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[1] Lund,T.C., Tolar,J., & Orchard,P.J. (2008) *Haematologica* 93:908-912,Submitted herewith as **Exhibit E.**

[2] Piersma,A.H., et al, (1985). *Cell Tissue Kinet* 18:589-595, Submitted herewith as **Exhibit F.**

suspension containing a population of disaggregated adipose-derived cells comprising adipose-derived stem cells, one could not arrive at Applicants' claimed compositions, which exhibit the unexpected benefits that could not have been predicted from the teachings of the state of the art, including Katz, Peterson, or any of the other references of record that discuss either populations of disaggregated adipose-derived cells alone, or unprocessed adipose tissue alone.

Applicants' surprising results regarding long term retention of transplants with claimed compositions were seen in humans in a clinical trial. (See, Fraser Decl. at ¶¶14-19). As such, Applicants' compositions exhibit unexpected benefits over compositions comprising disaggregated adipose-derived cells seeded on scaffolds/matrices, or unprocessed tissue alone, but also satisfy a long-felt unmet need in the field of cosmetic and reconstructive surgery.

In view of the foregoing comments, Applicants respectfully submit that the claimed compositions are not obvious in view of the art at the time of filing, and that the claimed mixture provides unexpected benefits for autologous fat transfer, meeting a long-felt unmet need.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, the Applicants are not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. The Applicants reserve the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that the Applicants have made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

In view of the above amendments and remarks, Applicants respectfully maintain that the claims are patentable and request that they be passed to issue. Applicants invite the Examiner to call the undersigned if any remaining issues may be resolved by telephone.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: July 23, 2008

By: _____

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EXHIBIT A

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Contents

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Transplantation of Purified Autologous Fat: A 3-Year Follow-Up Is Disappointing

Robert A. Ersek, M.D.

Austin, Texas

The idea of autologous fat microtransplants has recently resurfaced because of interest by the press. Past experiments have shown that small amounts of fat may be transplanted with an expected survival rate between 10 and 50 percent without ectodermis. A great fund of knowledge exists showing that skin grafts will survive quite dependably if their thickness is up to about 0.0020 inch. For the first week or so, they live by diffusion and inosculation, and then neovascularization enables them to continue viability.

I have developed a technique whereby fat is harvested through a blunt cannula with minimal vacuum to prevent explosion of the cellular fat globules. Fibrin and cellular debris are removed, and the fat globules are separated from the free fat, blood, and other constituents of the aspirate. Individual, free-floating 1- to 3-mm adipose fragments are then suspended in nutrient solution and injected through an 18-gauge needle. Multiple radial pathways, with each fat segment being separated from the other by host tissue, maximizes the host-prosthesis interface and the possibility for exchange of nutrients.

I have attempted this procedure in more than 100 patients with widely varying results. In acne pits of the face, no significant improvement could be noted 6 weeks after injection. For the first few weeks, a near-perfect result was obtained, but none of these transplants in scarred areas of this kind have been of benefit. Injection of 10 to 50 cc in other areas has resulted in some cells (perhaps 10 percent) surviving over 2 years. I do *not* recommend autologous fat injection as a permanent transplant at this time, but I am continuing investigations to improve survival. An anonymous survey of the opinions of my patients shows a low regard by them for the results.

Transplantation of autologous fat is an old idea that has recently gained new interest. To a large extent, this is due to applications of transplanted fat as a function of the improved understanding of the nature of subcutaneous fat resulting from refinements in the techniques of suction lipoplasty. If we can achieve good results in correcting contour defects by removal of gratuitous fat, this reasoning goes, then we should be able to achieve similar results by replacing fat in defects characterized by the reverse type of problem. It seems to be so simple.

The technique I use for blunt suction lipectomy is reasonable in terms of both safety and dependability of the results, as demonstrated by my success in a high percentage of widely varying cases previously reported.[1–4] I have observed consistently, however, that the fat I remove is accompanied by some surrounding tissue, fibrinogen, and dead cells. Hetter[5] has shown that the aspirate is about 25 percent blood. The composition of this fat after removal, therefore, differs from that in situ prior to suction lipectomy. I felt that I would optimize chances for survival of the fat on transplantation if I could restore it, as nearly as possible, to its original state. I report, in detail, on three typical patients in whom I have transplanted purified fat, with one success, one failure, and one minimal improvement. My 100 other patients were treated in the same manner.

Materials and Methods

Fat was harvested from convenient areas, such as the abdomen, thighs, or mons, using the blunt technique of suction lipectomy. The procedure is done under local anesthesia with sedation. The suction machine is set to below maximum or a 20-gauge hypodermic needle is inserted into the suction tubing to prevent negative pressure that would cause boiling of the fat with subsequent rupturing of cells. The fat is harvested using a 2- or 3-mm cannula. It is then agitated with a wire

From the Department of Plastic Surgery at the Southwest Texas State University. Received for publication November 23, 1987; revised March 12, 1990.

whisk (Fig. 1) in order to separate the fibrin and any broken cells and free lipids from the clumps of fat. The fat is then rinsed repeatedly with Eagle's medium to cleanse it of the separated impurities. The resulting clumps of cleansed fat are then suspended in physiologic solution (Tissol) and injected in the desired location using a 16- or 18-gauge blunt needle. The fat is injected in a multitude of narrow pathways, each separated from the other, so that no clumps of contiguous fat will compete for nutrients and each is surrounded by host tissue. This is consistent with my observation of the subcutaneous tissue structure surrounding the fat that I remove by liposuction, wherein I have learned by experience that any side-to-side motion of the cannula has the effect of breaking down support structures that, while barely noticeable when the fat is present, provide vital and irreplaceable support for the overlying skin after fat has been removed.

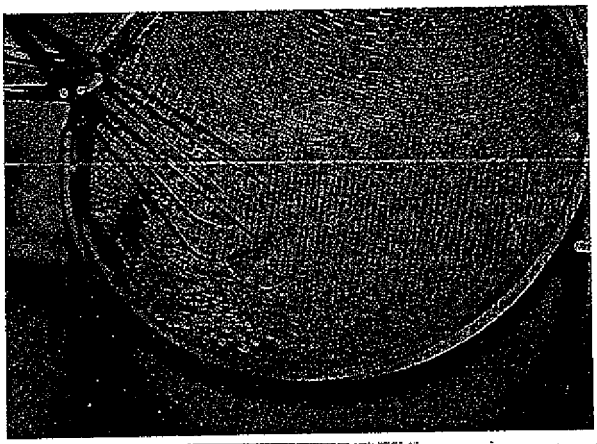

FIG. 1. Harvested fat is agitated with a wire whisk, separating the fibrin, broken cells, and free lipids from the clumps of fat, which are then rinsed to produce the cleansed fat that is seen collecting below the strainer.

SURVEY

In attempting to evaluate the results of autologous fat transplantation, I found it very difficult to compare preoperative and postoperative photographs, even though they were taken with the same camera, same light, and same distance and were often accompanied by a color chart that was printed with the colors selected by the wavelength of light so that the primary colors of yellow, red, and blue would be precisely comparable even years later. In looking at some of these defects and discussing them with the patients, I found it difficult to convince myself that the defects were substantially improved. Therefore, I sent an anonymous survey to each of my patients. I sent this survey anonymously so that the patients could respond without fear of hurting my feelings and could say anything without being identified. I was able to locate 41 of my patients who were treated more than 2 years previously. The total responding to the survey was 15. The questions and results are shown in Table I.

SURVEY RESULTS

I explained to every one of these patients receiving autologous fat microinjections the experimental nature of this procedure, and I did not charge them at any time for any of these fat injections, nor for their postoperative visits. I feel it is very disappointing that in this anonymous survey only 3 patients were satisfied with the results; 8 patients frankly said "no," and another 4 patients did not answer that question. All 15 patients did not have any postoperative complications that required a physician's attention.

TABLE I

Survey Results: Autologous Fat Transplant Patients

Total mailed: 41
Total responses: 15 (male, 2; female, 12; unknown, 1)

Questions	Yes	No	Unanswered
2. Are you satisfied with the results?	3	8	4
4. Did you have postoperative complications requiring a doctor's attention?	0	15	0
5. Would you recommend this procedure to a friend?	6	6	3
6. Do you believe the fat survived?	4	10	1
8. Complications listed below:			
Wrinkling	3		
Discoloration	5		
Numbness	1		
Increased sensitivity	0		
Other	2		

CASE REPORTS

Case 1

A 41-year-old woman who had been treated by me previously for an unrelated problem inquired about the possibility of dermabrasion to correct severe acne scars throughout her face (Fig. 2). She was advised of the limitations of dermabrasion in correcting severe pockmarks, and I suggested to her that fat transplantation might offer a better chance of producing the improvement in appearance she desired. She was informed of the experimental nature of this technique and was told that the outcome could not be ensured, but that dermabrasion would still be an option if the fat transplantation did not succeed. The patient agreed that she would try fat transplantation.

Fat was taken from the medial and lateral thighs by the blunt suction lipectomy technique and then strained and rinsed. Approximately 5 cc of the cleansed fat was then injected into the pockmark scars throughout the glabellar frown lines, wrinkles, cheeks, and chin using an 18-gauge needle. No pressure dressings were applied to the treated areas. Erythromycin and pain medication were prescribed.

Immediately following the procedure, it appeared that the patient's pockmark scars had been substantially improved, an appearance that persisted at 1 week postoperatively (Fig. 3). Over the course of the following 2 weeks, however, the treated pockmarks gradually reverted to near their original appearance, and by 23 days postoperatively, the transplanted fat appeared to have been almost completely absorbed (Fig. 4). Three months after transplantation, the patient's appearance showed virtually no change from that prior to the procedure (Fig. 5). The procedure was not repeated, and the patient has not elected to try dermabrasion at this writing.

Case 2

A 43-year-old woman presented with moderate regional lipodystrophy of the abdomen and thighs that she wished to have removed by liposuction. She also had a depressed scar on the left anterior calf from previous reconstructive surgery that she wished to have elevated (Fig. 6). She was informed that it might be possible to correct the scar depression by transplantation of a portion of the fat to be removed by liposuction. She was also told that this was an experimental procedure and that the results could not be ensured, but that there was little risk of any undesirable complications other than failure of the transplanted fat to survive.

Using the blunt suction lipectomy technique, fat was removed from the abdomen up to the ribcage from the lateral waist, hip area, and medial and lateral thighs through escutcheon incisions. The fat was strained and rinsed, and approximately 30 cc of the cleansed fat was injected into multiple radial channels in the depressed scar using a 16-gauge blunt needle. Immediately following the procedure, the depression appeared to be improved and "overcorrected." The scar area was wrapped with an Orthoplast splint to maintain pressure on the lateral borders of the scar while minimizing pressure on the central portion. After the suction wounds were closed, the patient was placed in a compression girdle and released. When seen 1 week after the procedure, the scar depression continued to show considerable improvement.

Although the improvement in the scar persisted unchanged over a period of several weeks, the indentation was soon noticeable. The patient requested a second injection of autologous fat for further improvement. Exactly 2 months following the initial procedure, 150 cc of fat was removed from the medial and lateral thighs by blunt suction lipectomy, and after straining and rinsing, approximately 60 cc of the fat was injected into the scar area in the same manner

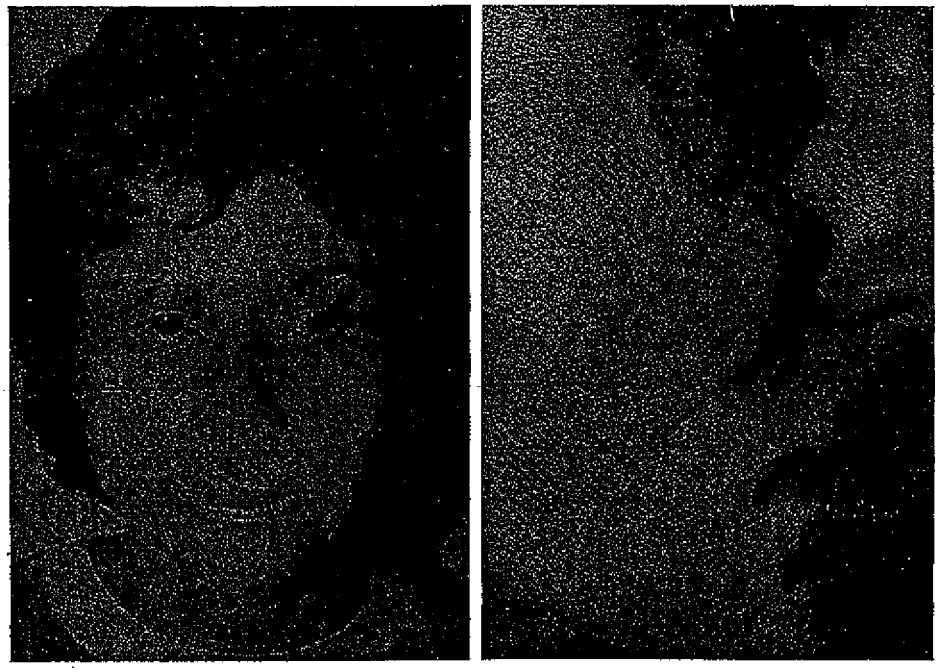

FIG. 2. *Case 1.* Patient as she appeared when she inquired about dermabrasion to improve acne scars.

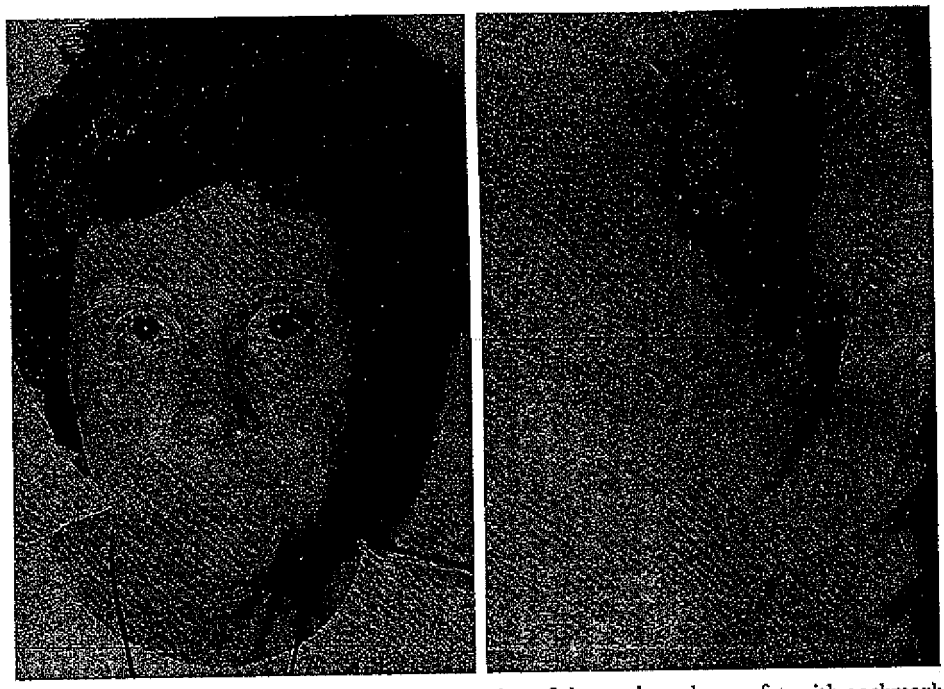

FIG. 3. *Case 1.* Patient 1 week after transplantation of cleansed autologous fat, with pockmark scars appearing to be improved.

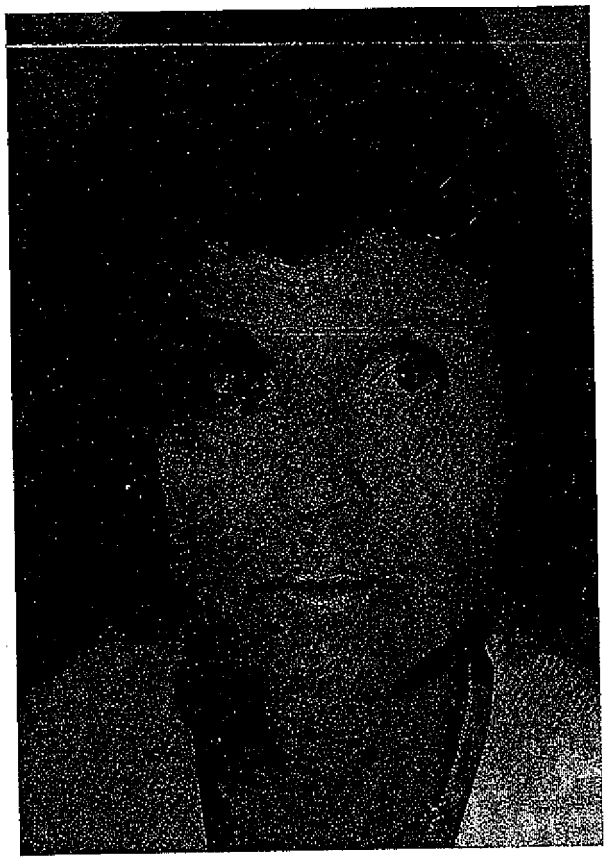

FIG. 4. *Case 1.* Patient 3 weeks after transplantation; the fat has been almost completely absorbed.

as before. There appeared to be immediate improvement in the scar depression, which was wrapped with a custom-made splint to reduce pressure in the central area.

Three weeks following the second implantation of fat to the scar depression (11 weeks after the initial implantation), the scarred area appeared to be about 95 percent filled out. There was slight irregularity in the surface of the scar, visible only when viewed from selected angles. The patient continued to wear the protective splint at night for an additional 3 weeks. At 9 months following the second fat transplantation, there had been no observable regression in the improved appearance of the scar (Fig. 7), and the patient was satisfied with its appearance and had no complaints about the area of the transplantation.

Three years later, there again appeared a depression over the entire length of the scar to near the preoperative level. The patient is convinced that there is some improvement because the scar is not adherent to the tibia, as it was prior to fat injections.

Case 3

A 22-year-old man presented who had been injured at age 2. When caught in a house fire, his forehead was severely burned. This was a deep, second-degree burn with areas of third-degree burn that resulted in an irregular contour to his forehead. This irregular contour was due to the difference in depth of burn and subsequent scarring and healing (Fig. 8). The patient inquired about the possibility of dermabrasion to these areas. Since the problem was not one of hills and valleys of normal skin, but due to actual deficiencies in these scarred areas, I described the procedure of autologous fat transplantation to him. With his understanding of the experimental nature of the procedure, I injected his forehead (Fig. 9) on two occasions. The immediate results were very pleasing, and he had a smooth contour. As the

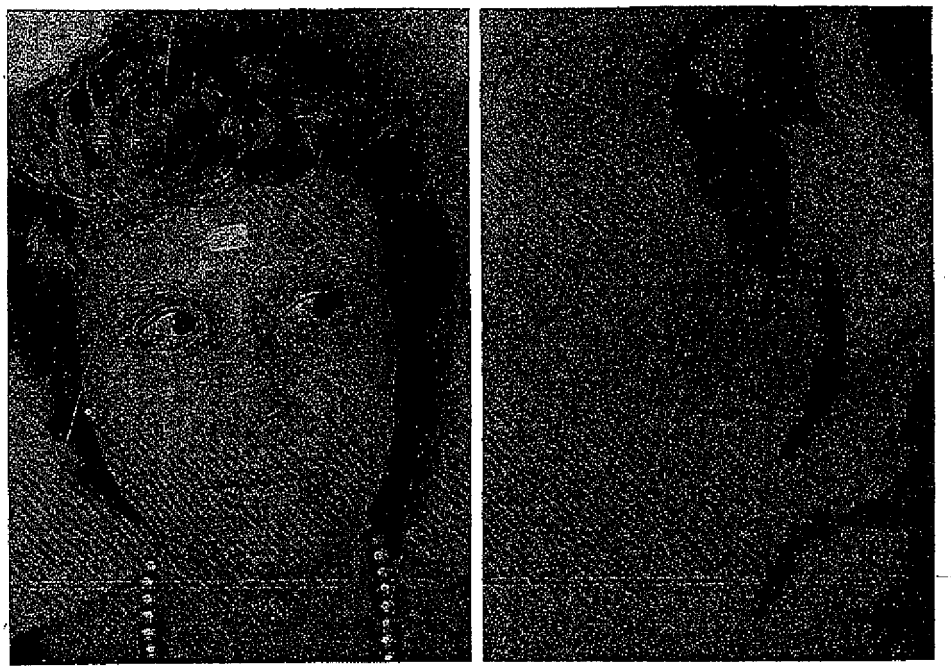

FIG. 5. *Case 1.* Patient 3 months after transplantation, with appearance virtually unchanged from that prior to the procedure.

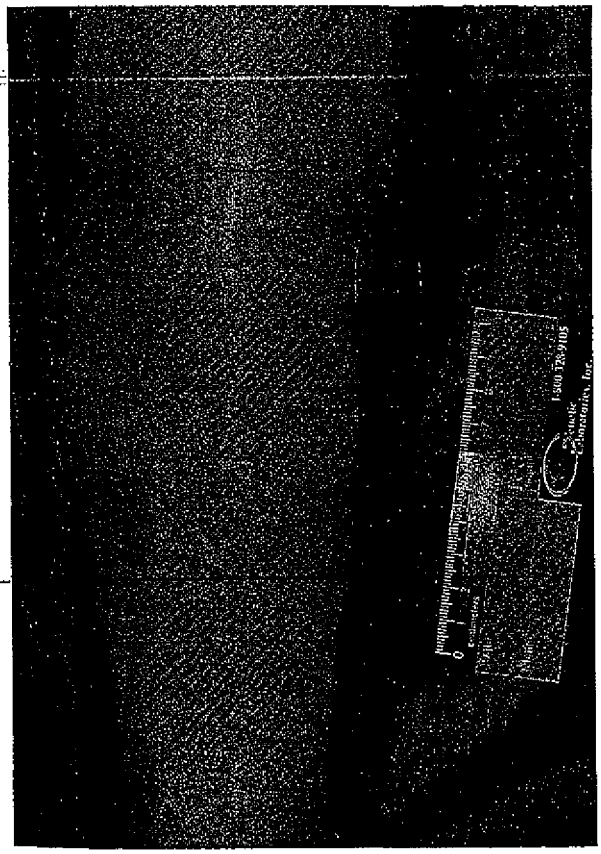

FIG. 6. *Case 2.* Patient showing the depressed scar on the left anterior calf.

months went by, however, some areas diminished in their improvement, and no areas remained perfect. After 1 year, because of persistence of the scar in the most irregular portion of his forehead, in the very center, he asked if I might remove this area by direct excision. In so doing, I was able to obtain a biopsy of the area where I had injected autologous fat into the previous scar. The photomicrograph in Figure 10 is a low-power view of a cross section of the forehead skin and scar. Within this scar, the collagen of the scar dermis can clearly be seen; islands or clumps of fat cells appear to be typical signet ring cells of living fat. No microcysts are seen, and at least in this instance, it does appear that some of this fat did persist. Subsequent follow-up 1 year later (2 years after the original autologous fat injection) showed minimal improvement in forehead contour (Fig. 11). The patient, however, insists that this is a substantial improvement, and he is pleased with the results.

DISCUSSION

Writing in the 1950s, Peer[6,7] reported that absorption of 50 percent or more of transplanted autologous fat could be anticipated in virtually all patients. Three decades later, Ellenbogen,[8] approaching the problem perhaps more thoroughly, reported similar findings and confirmed the conclusions of numerous authors in the intervening decades that smaller globules of fat experience higher rates of absorption. Chajchir and Benzaquen[9] reported satisfactory results in virtually all patients, but noted that they routinely transplant 50 percent more fat than they believe necessary for the desired result, in

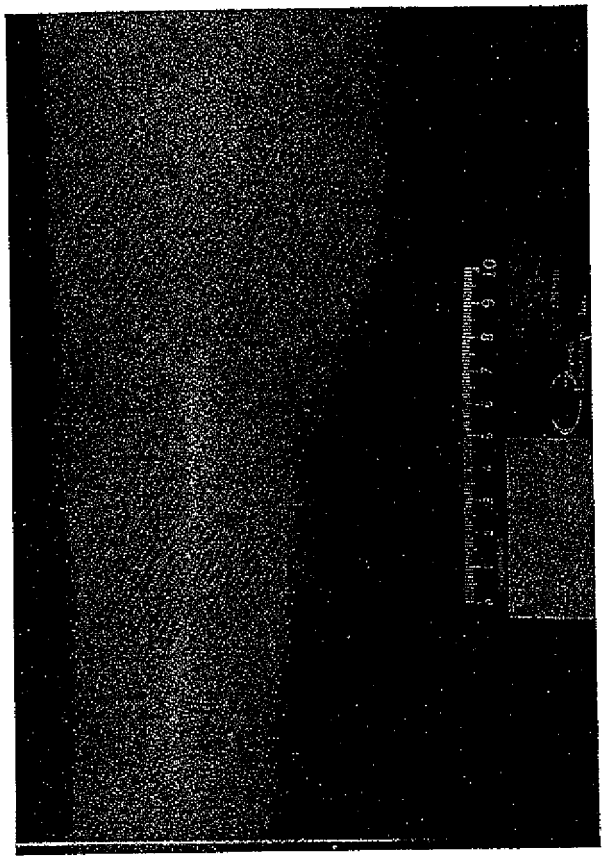

FIG. 7. *Case 2.* Patient showing the same scar area 11 months later, after transplantation of 90 cc of cleansed fat in two separate procedures. The appearance of the scar is substantially improved, with little change from that observed immediately following the second fat transplantation.

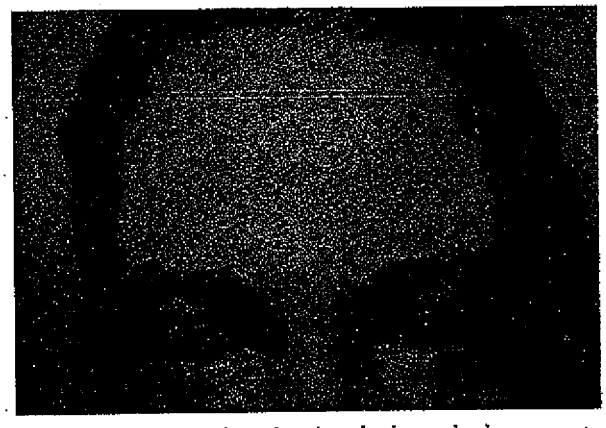

FIG. 8. *Case 3.* Patient showing the irregular burn scar to his forehead.

anticipation of 30 to 60 percent absorption. Ellenbogen[8] cut harvested fat into "pearls" and then bathed them in insulin. Chajchir and Benzaquen[9] emphasized cautious handling of the harvested fat to avoid crushing or other injury

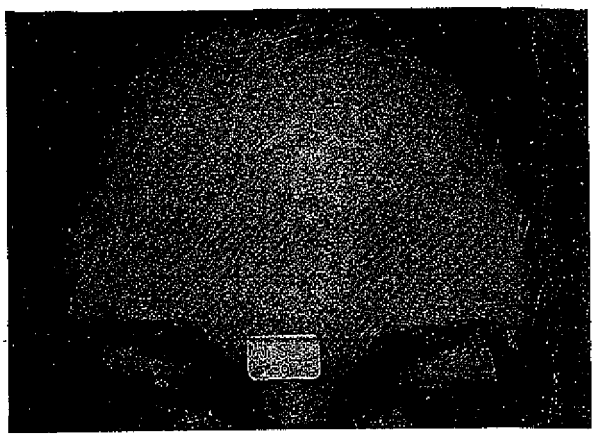

FIG. 9. *Case 3.* Patient showing the scar after two injections of autologous fat.

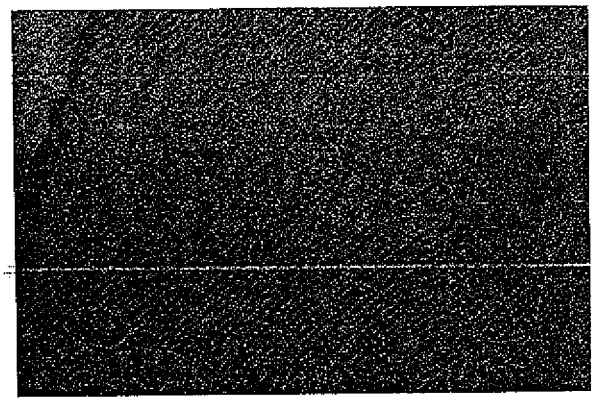

FIG. 10. *Case 3.* Low-power, cross-sectional photomicrograph of the forehead skin and scar removed from patient. Within the scar, the collagen of the scar dermis can be seen clearly. Clumps of fat cells appear to be typical signet ring cells of living fat.

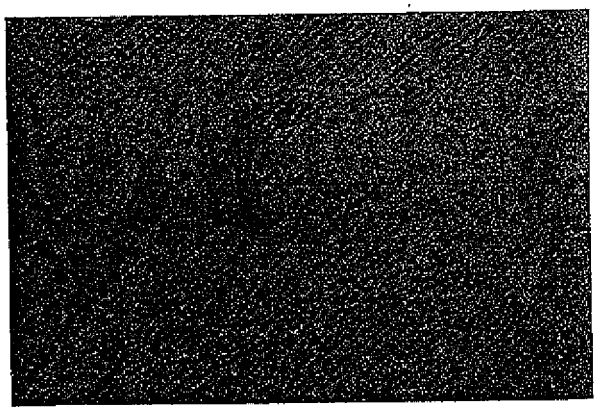

FIG. 11. *Case 3.* High-power, cross-sectiomal photomicrograph of forehead skin and scar removed from patient. Two years after original transplantation the patient showed minimal improvement.

that would interfere with graft survival. Sidman[10] reported injecting insulin into transplanted fat in vitro. All experienced results not greatly different from those reported by Peer, and despite their special efforts to minimize absorption, none appeared closer to an explanation for variations in graft survival.

In the three patients reported here, no extraordinary efforts were taken to protect the injected fat from absorption beyond the cleansing process described. By separating the fat from the fibrin that is unavoidably harvested along with it, I am, in effect, isolating fat globules from other material, just as Ellenbogen attempted to do by cutting his "pearls" of fat, although I suspect that my chemical approach may be more thorough than the cutting method. Ellenbogen felt that the pearls of fat, which measure 4 to 6 mm in diameter, produced increased surface contact and, hence, improved survival. I would hypothesize, however, that an equally important factor in survival would be the purity of the transplanted fat and the resulting uniformity of the contact between the injected material and the surrounding tissue. I attempt to enhance this effect further by injecting the fat in narrow, separated pathways comparable to the narrow pathways from which I remove fat in liposuction. If supporting structures are important to results in liposuction, it would seem that they might play an equally important role in the results of fat injection. By first cleansing the fat and then injecting it in these pathways, I have made a serious effort to replicate as closely as possible the subcutaneous structure that I have found upon removal of fat by liposuction.

How, then, can I explain the divergent results obtained in these three patients? The fat injected in each patient had been treated identically and was in the form of similar globules. Yet, in one patient the fat was apparently absorbed completely within 3 weeks, while in another the injected fat appears not to have undergone any significant absorption at all over a period of almost 1 year from initial transplantation. The principal differentiating factor in these patients appears to be the fact that the globules of fat were injected in one patient virtually singly to treat acne spot scars, while in the other, fat was injected in larger quantities and in considerably greater total amounts into what was, because of the types of scars involved, probably more normal subcutaneous tissue. Ellenbogen[8] has said that acne scar patients treated by fat transplantation routinely need additional procedures because of the difficulty in identifying and treating all scars in a single treatment. In my patient, however, it appeared immediately after surgery that virtually all scars had been injected. Two weeks later, it appeared that fat had been absorbed to a significant degree from all treated scars.

The problems and difficulties that have been encountered with autologous fat transplantation were well summarized in the report released in September of 1987, by the Ad Hoc Committee on New Procedures of the American Society of Plastic and Reconstructive Surgeons.[11] The report concludes that only 30 percent of injected fat can be expected to survive for 1 year. "Therefore, overcorrection is necessary when performing fat transplants. Unpredictable results are inherent in the procedure, since scar tissue contributes significantly to the eventual appearance."[11] The report also says that "harvested fat can be reinjected immediately into the site targeted for augmentation." It notes that "cellular debris" in the harvested fat may cause an inflammatory response that can be reduced by bathing with Tissol or Ringer's solution, but that inflammation may, in fact, be desirable in promoting formation of scar tissue "to fill the contour defect rather than the tenuous survivability of the fat cells."[11]

Unfortunately, it appears that the report, in attempting, quite appropriately, to point out the lack of solid evidence in support of any one technique, may oversimplify somewhat the evidence gathered to date. It may be that some of the improvement noted in my second patient is partially attributable to scar tissue formation, but there is then no explanation for the failure of the attempted correction in the other patient. I also note that the fat used in my patients was cleaned, in contrast to the report's conclusion that cleaning of fat reduces inflammation and thus the formation of scar tissue, which the committee believes is the principal source of any improvement. In addition, I have observed, as have others, that there appears to be some relationship between both the size of the fat globules and the total number of globules injected and the ultimate success of the procedure, a fact that the scar effect does not appear to help to explain.

What we are left with, then, is no more certainty about the problems and potentials of autologous fat injection than we started with, and very little more than existed prior to the release

of the ASPRS report. I concur completely with the American Society of Plastic and Reconstructive Surgeons that this is very much an experimental technique, one that must be pursued with extreme caution. I note also that there can be no argument whatever with the report's condemnation of the use of fat transplantation in breast augmentation. Weber's preliminary report[12] on his work with transplantation of plugs of fat in rabbits indicates a bare 10 percent survival rate with definite evidence of calcification that could easily inhibit early diagnosis of breast tumors.

My disappointing results, even with repeated injections, lead me to the conclusion that very little, if any, of this autologous fat survives in its new site. It appears that Weber's 10 percent survival rate may be the most one can hope for with the current state of the art. If this is a valid concept, then filling a 1-cc defect might require 10 injections at 10 different times involving 1 cc of autologous fat each time. This might be a reasonable price to pay if long-term results could be ensured. However, in carefully reviewing the photographs of the 41 patients whom I have documented, I was unable to show a persistent, perfect result after 2 years. Perhaps the only positive thing that can be gained from this experience is that all the patients did not have any postoperative complications that required a physician's attention.

I was quite surprised that although only four patients thought that any fat survived, and only three were satisfied with the results, twice that number, six patients, would recommend the procedure to a friend. I can only surmise that they would recommend the procedure to a friend because it did not cost anything, did not make things worse, and might have helped.

Ellenbogen states that "with all the fat tissue being discarded in present-day surgical procedures and all the aesthetic defects secondary to subcutaneous tissue loss, free fat should be considered as a replacement."[8] While it is difficult not to sympathize with this sentiment when faced with numerous contour defects and limited options for tissue replacement, it is also difficult to feel overly optimistic in the present situation. Autologous fat transplantation is a technique with too much potential to be discarded out of hand at this point. However, it is also too early, and the preliminary results are too tenuous, to have any confidence about the future of autologous fat transplantation.

Fredricks' discussion[13] of injection fat grafting for soft-tissue augmentation by Chajchir and Benzaquen argues that the "emotional and financial investments of the patient are not justified for a short-lived improvement that tantalizes and ultimately disappears. The profession certainly is in need of a readily available, inexpensive, nonallergic, injectable bulking material that gives a long-lasting improvement. In my opinion, we have not as yet found that material, and autologous fat is certainly transitory and not the answer."[13]

While further efforts to define transplantation methods should be encouraged, my discouraging results with viable autologous fat transplant methods are leading me in other directions. It appears that an inert substance that could be implanted by injection surgery and is biocompatible and nondegradable may be more easily achieved at the present level of biomedical engineering.

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Discussion

Transplantation of Purified Autologous Fat: A 3-Year Follow-Up Is Disappointing

by Robert A. Ersek, M.D.

Discussion by Simon Fredricks, M.D.

I read with interest the 3-year experience of Dr. Ersek with autologous fat grafts. The experience of the six surgeons of Houston Plastic Surgery Associates has been similar.

I have followed the exact regimen advocated by Dr. Ersek with the same disappointing results, except in two patients. In these two patients, the purified autologous fat was placed in the hip and in the anterior thigh, respectively. In these two areas, a permanent improvement of contour depression has lasted over 1 year, and therefore, I believe it will be permanent. It is therefore my impression that fat transplantation may be more permanent in extremities as opposed to the face.

Everyone recommends avoiding trauma to the transplanted fat cells. I recommend, therefore, that cleansed and purified material be placed into the end of the barrel of a syringe to which a very large bore intracath needle is attached, e.g., 12 gauge. Then the needle with the metal core in place is inserted into the distal-most point of desired augmentation. The metal needle is then withdrawn, and the autologous fat laid down through the Teflon outer needle only as it is withdrawn. The metal needle is then reinserted, and the process is repeated as often as required until the desired fat augmentation is accomplished. I believe that the resistance to fat injection is thereby dramatically reduced and fat cell injury is markedly prevented.

I compliment Dr. Ersek on his paper and conclude also that fat transplantation is to date a much heralded but generally disappointing procedure.

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EXHIBIT B

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Comparison of the Release of Adipokines by Adipose Tissue, Adipose Tissue Matrix, and Adipocytes from Visceral and Subcutaneous Abdominal Adipose Tissues of Obese Humans

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The purpose of this study was to examine the source of adipokines released by the visceral and sc adipose tissues of obese humans. Human adipose tissue incubated in primary culture for 48 h released more prostaglandin E_2, IL-8, and IL-6 than adiponectin, whereas the release of plasminogen activator inhibitor 1 and hepatocyte growth factor was less than that of adiponectin but greater than that of leptin. IL-10 and TNFα were released in amounts less than those of leptin, whereas vascular endothelial growth factor and IL1-β were released in much lower amounts. The accumulation of adipokines was also examined in the three fractions (adipose tissue matrix, isolated stromovascular cells, and adipocytes) obtained by collagenase digestion of adipose tissue. Over 90% of the adipokine release by adipose tissue, except for adiponectin and leptin, could be attributed to nonfat cells. Visceral adipose tissue released greater amounts of vascular endothelial growth factor, IL-6, and plasminogen activator inhibitor 1 compared with abdominal sc tissue. The greatly enhanced total release of TNFα, IL-8, and IL-10 by adipose tissue from individuals with a body mass index of 45 compared with 32 was due to nonfat cells. Furthermore, most of the adipokine release by the nonfat cells of adipose tissue was due to cells retained in the tissue matrix after collagenase digestion. (*Endocrinology* 145: 2273–2282, 2004)

RECENTLY, THE CONCEPT of adipose tissue as an endocrine organ has become accepted (1–5). Leptin and adiponectin are proteins secreted by the adipocytes of adipose tissue, and there is an inverse relationship between circulating adiponectin and leptin (6). These hormones are often referred to as adipokines because they are secreted by the adipocytes of adipose tissue. A variety of other factors are also released by adipose tissue *in vitro* including cytokines such as IL-6 (7) and IL-8 (8), which when released by adipose tissue have been called adipocytokines or adipokines. We use the term adipokine for any substance released by adipose tissue. Although leptin and adiponectin are released to the blood as hormones, other adipokines appear to be paracrine factors whose release by adipose tissue may not contribute to circulating levels.

The concentration in blood of many adipokines, hormones, and acute-phase proteins is altered in human obesity. Leptin is elevated, whereas plasma adiponectin is reduced in obese humans (1–6). C-reactive protein (CRP) (9–15) is an example of an acute-phase protein whose circulating level is higher in obese than in nonobese individuals. Blood levels of IL-10 (15), IL-6 (11–19), IL-8 (18, 20), plasminogen activator inhibitor 1 (PAI-1) (17, 21–24), TNFα (11, 25), and hepatocyte growth factor (HGF) (26) have all been reported to be elevated in obesity. There is some controversy about circulating levels of TNFα because several groups found either no detectable TNFα (27) or no increase of circulating TNFα in obesity (10, 18, 19, 28). However, there was greater release of TNFα by adipose tissue (27, 28) as well as elevated levels of TNFα mRNA in adipose tissue from obese humans (27). Furthermore, the studies of Mohamed-Ali *et al.* (29) indicated that there was release of IL-6 but not of TNFα by sc adipose tissue *in vivo*.

Previously, we demonstrated that substantial amounts of connective tissue and blood vessels remain after collagenase digestion of human adipose tissue (30). We describe this fraction as tissue matrix, and it accounts for almost 70% of the total protein retrieved in the three fractions obtained by collagenase digestion of human adipose tissue. The remainder of the protein was equally divided between the isolated stromovascular (SV) cells that do not contain enough lipid to float and adipocytes that do float. Although there was no appreciable release of leptin by the tissue matrix or the SV cells, these two fractions accounted for over 95% of the prostacyclin or prostaglandin E_2 (PGE_2) released over a 48-h incubation per gram of adipose tissue (30). Similar results were seen with respect to resistin release (31). The hypothesis we wanted to examine was whether the source of other adipokines, which are defined as factors released by adipose tissue, is the adipocytes or the nonfat cells present in adipose tissue. The first aim was to compare adipokine release by tissue with that by adipocytes, the isolated SV cells, and the undigested adipose tissue matrix of human adipose

Abbreviations: BMI, Body mass index; CRP, C-reactive protein; HGF, hepatocyte growth factor; PAI-1, plasminogen activator inhibitor 1; PGE_2, prostaglandin E_2; SV, stromovascular; VEGF, vascular endothelial growth factor.

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tissue. The second aim was to examine the time course for the release of adipokines by human sc and visceral adipose tissue explants and adipocytes in primary culture from morbidly obese [body mass index (BMI) >40] individuals. The third aim was to compare release by tissue and adipocytes from individuals over 48 h with an average BMI of 45 *vs.* that by tissue and adipocytes from individuals with an average BMI of 32. The fourth aim was to compare the relative release of adipokines by visceral compared with sc adipose tissue.

Materials and Methods

Abdominal sc and visceral adipose tissue were obtained from women who were undergoing open abdominal surgery (abdominoplasty) or who were undergoing laparoscopic gastric bypass with Roux-en-Y gastroenterostomy surgery for the treatment of morbid obesity. Body fat content was determined using bioelectrical impedance (Tanita TBF-310GS Body Composition Analyzer/Scale, Tanita Corp., Arlington Heights, IL). Each experimental replication involved tissue from a separate individual. The study had the approval of the local institutional review board, and all patients involved gave their informed consent. The patients were on a clear liquid diet the day before surgery, but they had not been on any type of dietary restriction before surgery.

Samples of visceral and abdominal sc adipose tissue were immediately transported to the laboratory. The handling of tissue and cells was performed under aseptic conditions. The tissue was cut with scissors into small pieces (10–20 mg). All the studies used explants of adipose tissue that had been incubated in buffer plus albumin (3 ml/g of tissue) for approximately 30 min to reduce contamination of the tissue with blood cells and soluble factors. At the conclusion of the 30-min incubation, the tissue explants were centrifuged for 30 sec at 400 × *g* to remove blood cells and pieces of tissues containing insufficient adipocytes to float. The explants were separated from the medium plus the sedimented cells and resuspended in fresh buffer. The explants (500 mg/5 ml) were then incubated in duplicate for 48 h in suspension culture under aseptic conditions.

One gram of cut tissue, again in duplicate, was incubated in 2 ml of incubation medium containing 1.3 mg of bacterial collagenase in a rotary water bath shaker (100 rpm) for 2 h. The collagenase digest was then separated from undigested tissue matrix by filtration through 200-μm mesh fabric. Five milliliters of medium were then added back to the digestion tubes and used to wash the undigested matrix on the filter mesh. This wash solution was combined with the collagenase digest, and SV cells were separated from adipocytes and medium by centrifugation in 15-ml tubes for 1 min at 400 × *g*. The SV cells are defined as those cells isolated by collagenase digestion that do not float. The SV cells and adipocytes were each suspended in 5 ml of fresh buffer and centrifuged for 10 sec at 400 × *g*. The medium was removed. The undigested tissue matrix on the nylon mesh, the SV cells, and the adipocytes were then incubated in a volume of 5 ml for the indicated periods.

The serum-free buffer for incubation of adipose tissue and adipocytes was as previously described (30, 31). The pH of the buffer was adjusted to 7.4 and then filtered through a 0.2-μm filter. Aliquots of the medium were taken and stored at −20 C for measurement of release to the medium. Leptin and adiponectin were determined on all samples using RIA kits from Linco Research, Inc. (St. Charles, MO) and by ELISA using reagents from R&D Systems, Inc. (Minneapolis, MN). Lipolysis was measured as glycerol (32). Lactate was measured using lactate dehydrogenase and PGE_2 as described by Parfenova *et al.* (33). IL-6, IL-8, IL-10, TNFα, HGF, and IL-1β were measured using ELISA kits from the Central Laboratory of The Netherlands Red Cross that are distributed by Research Diagnostics (Flanders, NJ) or DuoSet ELISA development kits from R&D Systems, Inc. PAI-1 and vascular endothelial growth factor (VEGF) were measured using ELISA kits from American Diagnostica (Greenwich, CT) and Pierce Biotechnology (Rockford, IL), respectively.

All values are shown as the mean or the mean ± SEM. Pearson correlation coefficients were determined using the GraphPad Prism Program (GraphPad Software, Inc., San Diego, CA) assuming a Gaussian population and a two-tailed *P* value.

BSA powder (Bovuminar, containing <0.05 mol of fatty acid/mol of albumin) was obtained from Intergen (Purchase, NY). Bacterial collagenase *Clostridium histolyticum* (type I) was obtained from Worthington Biochemical Corp. (Lakewood, NJ; lot CLS1-4197-MOB3773-B, 219 U/mg). Other chemicals were from Sigma Chemical (St. Louis, MO).

Results

Release at 4 h vs. that at 48 h by tissue explants and adipocytes

In the studies shown in Table 1, we examined the time course for release of adipokines by tissue explants from 12 obese individuals with an average BMI of 42. Comparison of release on a molar basis indicated that 25-fold more PGE_2, 5-fold more IL-8, and 2-fold more IL-6 than adiponectin was released by adipose tissue explants over the first 4 h of incubation in serum-free medium containing 1% albumin. Leptin release was 10% of that seen for adiponectin over 4 h. PAI-1 and HGF were released in amounts greater than those of leptin, whereas IL-10 and TNFα were released in smaller amounts than of leptin. Even smaller amounts of VEGF and IL-1β were released over 4 h.

We also compared the release of adipokines to the medium by explants of human adipose tissue over 24 and 48 h (Table 1). The most striking difference was the marked decrease in TNFα release between 4 and 48 h. In fact, there was a net disappearance of TNFα because total release over 24 h was 180 fmol/g, whereas release over the first 4 h was 201 fmol/g. Adiponectin release per 4 h over 24

TABLE 1. Comparison of the release of adipokines and other factors at 4 h with that at 24 and 48 h by human adipose tissue

Adipokine	4-h value (fmol/g·4 h)	0- to 24-h value (fmol/g·4 h)	0- to 48-h value (fmol/g·4 h)
PGE_2	152,000 ± 40,000	108,000 ± 16,000	244,000 ± 38,000
IL-8	30,400 ± 16,000	43,100 ± 2,200	53,000 ± 11,500
IL-6	14,600 ± 2,600	21,300 ± 3,500	18,100 ± 440
Adiponectin	6,100 ± 1200	1,390 ± 280[c]	705 ± 140[c]
PAI-1	1,660 ± 220	7,310 ± 490[c]	14,570 ± 1575[c]
HGF	746 ± 89	323 ± 122[b]	192 ± 20[c]
Leptin	595 ± 138	230 ± 44[b]	275 ± 38[a]
TNFα	201 ± 17	30 ± 1[c]	8 ± 1[c]
IL-10	194 ± 27	265 ± 27	157 ± 22
VEGF	22 ± 2	38 ± 11	22 ± 4
IL-1β	18 ± 3	26 ± 6	15 ± 2

Explants (100 mg/ml) were incubated for 48 h from 12 individuals with an average BMI of 42. The values are shown as the mean ± SEM of 24 experiments equally divided between visceral and sc adipose tissue explants in which 4-, 24-, and 48-h values were compared on explants from the same individual. Adipokine release to the medium is in fmol/g·4 h. Statistically significant differences between release over 0–24 h or 0–48 h as compared with that during 0–4 h are indicated as follows: [a]$P < 0.05$; [b]$P < 0.025$; [c]$P < 0.001$.

and 48 h was only 23% and 12%, respectively, of that seen over the first 4 h. The release of leptin per 4 h over 48 h was 46% of that during the first 4 h, whereas that of HGF was 26% of that seen during the first 4 h. There were statistically insignificant changes in the rates of PGE_2, IL-8, IL-6, IL-10, VEGF, and IL-1β release per 4 h over 48 h. In contrast, there was a substantial (>8-fold) up-regulation of PAI-1 release between 4 and 48 h of incubation.

The time course for release by human adipocytes of adipokines at 4, 24, and 48 h is shown in Table 2. Data for IL-10 release are not shown because its release was so low during the first 4 h that it could not be accurately determined. Human adipocytes released at least 3-fold more PGE_2 over the first 4 h of incubation than of adiponectin (Table 2). The accumulation of IL-8 in the medium was only slightly less than that of adiponectin at 4 h but substantially higher over 48 h because of the up-regulation of its release by adipocytes between 4 and 48 h (4.7-fold). Leptin release was only 6% of that for adiponectin over the first 4 h. PAI-1 was released in amounts only slightly less than those of leptin over 4 h but there was a 5.5-fold increase in PAI-1 accumulation per 4 h over 4–48 h. HGF, VEGF, TNFα, and IL-1β were released in amounts far less than those of leptin and their release was down-regulated over 4–48 h. Leptin release by adipocytes was up-regulated over 4–48 h, whereas adiponectin release was down-regulated. Up- or down-regulation are used here to refer to changes in the net accumulation of adipokines in the medium that could involve changes in the rate of release as well as that of degradation or both.

The major difference in adipokine release between 4 and 48 h in adipocytes *vs.* explants was the up-regulation of leptin release in adipocytes compared with down-regulation in explants (Tables 1 and 2). The release of PAI-1 was up-regulated between 4 and 48 h in both adipocytes and adipose tissue explants, whereas that of adiponectin, TNFα, and HGF was down-regulated to about the same extent in tissue explants and isolated adipocytes. Minor differences were the greater up-regulation of PGE_2 and IL-8 release over 4–48 h in adipocytes as contrasted with explants as well as down-regulation of VEGF release in adipocytes but not in adipose tissue explants.

Effect of BMI on adipokine release by adipocytes and tissue explants from visceral and sc adipose tissue

We compared the release of 11 adipokines as well as that of glycerol and lactate over 48 h of incubation by visceral adipose tissue explants and adipocytes from eight gastric bypass subjects with an average BMI of 45 and eight abdominoplasty subjects with an average BMI of 32. There was no difference between the age or fasting blood glucose of the two groups of patients (Table 3). However, the abdominoplasty patients had a significantly lower BMI (−29%), waist measurement (−19%), weight (−26%), and total body fat mass (−43%).

Leptin, PAI-1, adiponectin, and VEGF release over 48 h were significantly greater by visceral adipose tissue explants expressed per gram of adipose tissue from individuals with a BMI of 32 compared with that of explants from subjects with a BMI of 45 (Table 4). The Pearson correlation coefficients were calculated for release of PAI-1, adiponectin, and VEGF by visceral adipose tissue explants *vs.* BMI for all 16 subjects shown in Table 4. For PAI-1 the correlation coefficient was −0.55 ($P = 0.03$), for adiponectin it was −0.81 ($P = 0.001$), and for VEGF it was −0.77 ($P = 0.001$). There were no significant differences in release of other substances per gram of tissue except for lactate formation by tissue explants, which was greater in visceral adipose tissue from individuals with a BMI of 32.

The release of adipokines by visceral adipocytes was much lower than that by explants of visceral adipose tissue (Table 4). However, the reduced release of adiponectin, PAI-1, and VEGF at a BMI of 45 compared with 32 was also seen in adipocytes. For visceral adipocytes, the correlation coefficient was −0.56 ($P = 0.023$) for adiponectin, −0.48 ($P = 0.06$) for VEGF, and −0.55 ($P = 0.03$) for PAI-1 accumulation *vs.* BMI of the fat donors.

Data for adipokine release by sc adipose tissue and adipocytes from the same individuals are shown in Table 5. The data differed from those by visceral adipose tissue explants (Table 4) where TNFα, IL-8, and IL-10 release were unaffected by BMI. However, TNFα, IL-10, and IL-8 release were markedly higher by sc adipose tissue explants from subjects with at a BMI of 45 compared with explants from individuals with a BMI of 32. PAI-1 release was unaffected by BMI in sc adipose tissue explants (Table 5), whereas it was elevated in

TABLE 2. Comparison of the release of adipokines and other factors at 4 h with that at 24 and 48 h by human adipocytes

Adipokine	4-h value (fmol/g·4 h)	0- to 24-h value (fmol/g·4 h)	0- to 48-h value (fmol/g·4 h)
PGE_2	4,800 ± 1100	5,050 ± 1300	10,550 ± 2100[b]
IL-8	1,100 ± 500	6,600 ± 1200[c]	5,200 ± 1500[b]
IL-6	705 ± 215	505 ± 95	375 ± 85
Adiponectin	1,445 ± 145	525 ± 85[c]	270 ± 30[c]
PAI-1	200 ± 22	533 ± 200	1,110 ± 770
HGF	35 ± 9	11 ± 3[b]	10 ± 3[b]
Leptin	233 ± 50	181 ± 38	364 ± 34[a]
VEGF	6 ± 1	5 ± 1	2 ± 1[c]
TNFα	5.0 ± 0.1	2.0 ± 0.1[c]	0.5 ± 0.2[c]
IL-1β	2.9 ± 1.3	1.3 ± 0.4	0.4 ± 0.2

Adipocytes (100 mg/ml) were incubated for 48 h from individuals with an average BMI of 42. The values are shown as the mean ± SEM of 18 experiments equally divided between visceral and sc adipose tissue adipocytes in which 4-, 24-, and 48-h values are compared on adipocytes from the same individual. Adipokine release to the medium is in fmol/g·4 h. Statistically significant differences between release over 0-24 h or 0-48 h as compared with that during 0-4 h are indicated as follows: [a]$P < 0.05$; [b]$P < 0.025$; [c]$P < 0.001$.

visceral adipose tissue explants from individuals with a BMI of 32 compared with 45. Adipocytes from sc adipose tissue differed from visceral adipocytes with respect to effects of BMI because release of PGE_2 was significantly higher and TNFα lower in sc adipocytes from individuals with a BMI of 32 (Table 5).

Comparison of adipokine release by visceral compared with sc adipose tissue explants

The statistically significant differences between release of adipokines by explants of sc compared with that by explants of visceral adipose tissue from subjects with an average BMI of 32 are shown in Fig. 1 based on paired comparisons. Visceral adipose tissue explants released more VEGF, IL-6, PAI-1, and PGE_2 than did explants of sc adipose tissue. However, the differences in release between sc *vs.* visceral adipocytes were small except for PAI-1, and none were statistically significant (Fig. 1).

Comparison of adipokine release by tissue matrix, SV cells, and adipocytes

The much smaller formation of most adipokines by adipocytes as contrasted to tissue explants from the same individuals (Tables 4 and 5) suggested that either collagenase digestion has deleterious effects on adipokine formation or there is much less formation by adipocytes than by the other cells present in adipose tissue. To correct for effects of collagenase digestion, we compared the release of adipokines as well as those of lactate and glycerol by the cells of the tissue matrix, the cells liberated by collagenase digestion passing through a 200-μm mesh filter that do not float (SV cells) and adipocytes. The data are uncorrected for breakage of adipocytes during collagenase digestion or for losses during the isolation and washing of the cell tissue digest. Recovery in the three fractions obtained after collagenase digestion of adipokine release ranged from 73–85% for leptin, IL-8, PGE_2, VEGF, and lactate formation. IL-1β accumulation over 48 h by the three fractions was 107%, and TNFα was 118% of that by tissue, suggesting some up-regulation, whereas that of glycerol was 64%, and IL-6 was 61%, suggesting some reduction in their formation or increased degradation due to collagenase treatment. The recovery of adiponectin, HGF, IL-10, and PAI-1 ranged between 45 and 51% indicating relative loss of their net accumulation as a result of collagenase digestion.

The data in Table 6 also indicate that leptin is made only by mature adipocytes that float. In contrast, lipolysis, which is based on glycerol release and immunoreactive adiponectin release, occurred at levels 94 and 64% of that by the nonfat cells, suggesting that cells in the adipose tissue matrix carry out some lipolysis and release adiponectin. PAI-1 was released by adipocytes in amounts that were 25% of that by the nonfat cells, whereas lactate formation by adipocytes was 16% of that by matrix plus SV cells. IL-8 formation by adipocytes was 12% of that by the matrix plus SV cells, whereas formation of TNFα, VEGF, IL-6, PGE_2, IL-1β, HGF, or IL-10 by adipocytes was 8% or less than that by matrix plus SV cells.

The question of whether comparing the release of adipokines by matrix, SV cells, and adipocytes was biased by measurements made at 48 h was examined in another series of experiments. We compared the formation of adipokines at 24 and 48 h by the adipose tissue matrix, SV cells, and adipocytes with

TABLE 3. Comparison of abdominoplasty and gastric bypass patients

Parameter	Abdominoplasty patients		Gastric bypass patients	
	Range	Mean ± SEM	Range	Mean ± SEM
Age (yr)	23–54	40 ± 4	29–56	40 ± 4
BMI	27–36	32 ± 1	41–48	45 ± 1^b
Waist (cm)	82–112	99 ± 4	112–138	122 ± 2^b
Weight (kg)	68–115	87 ± 6	93–133	117 ± 5^a
Fat (kg)	16–53	32 ± 4	41–70	56 ± 4^b
Blood glucose (mM)	4.4–6.4	5.0 ± 0.2	4.3–8.5	5.8 ± 0.5

The visceral and sc adipose tissue was obtained from eight females at the time of abdominoplasty (all Causasian) and eight females five Caucasian, one Hispanic, and two African-Americans) at the time of laparoscopic gastric bypass. The mean values ± SEM and the range of values are shown for each group and significant differences between the two groups are indicated as follows: $^aP < 0.005$; and $^bP < 0.001$.

TABLE 4. Comparison between the release of adipokines by explants of human visceral adipose tissue and isolated adipocytes as affected by BMI

	BMI of 45 (56 kg of fat)		BMI of 32 (32 kg of fat)	
	Tissue	Adipocytes	Tissue	Adipocytes
PGE_2	2300 ± 665	60 ± 42	3935 ± 710	136 ± 48
IL-8	2063 ± 310	72 ± 29	1594 ± 912	94 ± 29
PAI-1	182 ± 31	4.9 ± 2.3	412 ± 52^c	48 ± 14^b
Adiponectin	7.2 ± 1.2	2.0 ± 0.3	32 ± 9^a	7.2 ± 1.5^b
IL-6	141 ± 24	4.2 ± 2.1	188 ± 17	6.5 ± 1.3
Leptin	1.0 ± 0.4	0.67 ± 0.10	3.3 ± 0.5^a	3.2 ± .5^a
HGF	5.3 ± 0.6	0.07 ± 0.02	7.8 ± 1.3	0.08 ± 02
IL-10	1.4 ± 0.3	0.02 ± 0.01	1.0 ± 0.2	0.03 ± .01
TNFα	0.16 ± 0.03	0.01 ± 0.01	0.11 ± 0.02	0.01 ± 0.01
VEGF	0.28 ± 0.06	0.01 ± 0.01	1.00 ± 0.26^b	0.03 ± .01^b
IL-1β	0.18 ± 0.02	0.01 ± 0.01	0.23 ± 0.6	0.01 ± 0.01
Glycerol	6.4 ± 0.9	2.5 ± 0.4	10.4 ± 2.3	2.5 ± 1.0
Lactate	31 ± 4	3.3 ± 0.5	42 ± 2^a	4.1 ± 1.4

Difference between BMI of 45 and 32: $^aP < 0.05$; $^bP < 0.025$; $^cP < 0.001$.

Explants or adipocytes were incubated in primary culture for 48 h. The release of factors to the medium is in pmol/g except for glycerol and lactate that are in micromoles per gram. The data are mean ± SEM for adipose tisue and adipocytes from eight individuals with a mean BMI of 45 and eight with a BMI of 32.

TABLE 5. Comparison between the release of adipokines by explants of human sc adipose tissue and isolated adipocytes as affected by BMI

	BMI of 45 (56 kg of fat)		BMI of 32 (32 kg of fat)	
	Tissue	Adipocytes	Tissue	Adipocytes
PGE_2	1875 ± 225	37 ± 11	2100 ± 284	240 ± 57[c]
IL-8	2468 ± 257	87 ± 27	718 ± 99[c]	96 ± 27
PAI-1	137 ± 20	7.5 ± 2.7	142 ± 30	23 ± 7
Adiponectin	9.0 ± 1.7	2.7 ± 0.8	35 ± 15	4.8 ± 0.8
IL-6	82 ± 11	3.6 ± 1.1	60 ± 6	6.3 ± 1.4
Leptin	2.2 ± 0.6	1.1 ± 0.3	3.7 ± 1.2	2.1 ± 0.4
HGF	3.6 ± 2.0	.09 ± 0.01	6.1 ± 1.3	0.13 ± 0.5
IL-10	1.5 ± 0.3	0.01 ± 0.01	0.5 ± 0.1[b]	0.02 ± 0.01
TNFα	0.37 ± 0.05	0.03 ± 0.01	0.15 ± 0.02[c]	0.01 ± 0.01[b]
VEGF	0.15 ± 0.02	0.01 ± 0.01	0.21 ± 0.02[a]	0.03 ± 0.01
IL-1β	0.24 ± .03	0.01 ± 0.01	0.25 ± 0.06	0.01 ± 0.01
Glycerol	8.1 ± 0.9	3.0 ± 0.4	6.6 ± 1.0	1.6 ± 0.3[c]
Lactate	30 ± 3	4.0 ± 1.0	30 ± 5	4.0 ± 1.0

Difference between BMI of 45 and 32: [a]$P < 0.05$; [b]$P < 0.025$; [c]$P < 0.001$.

Explants or adipocytes were incubated in primary culture for 48 h. The release of factors to the medium is in pmol/g except for glycerol and lactate that are in micromoles per gram. The data are mean ± SEM for adipose tisue and adipocytes from eight individuals with a mean BMI of 45 and eight with a BMI of 32.

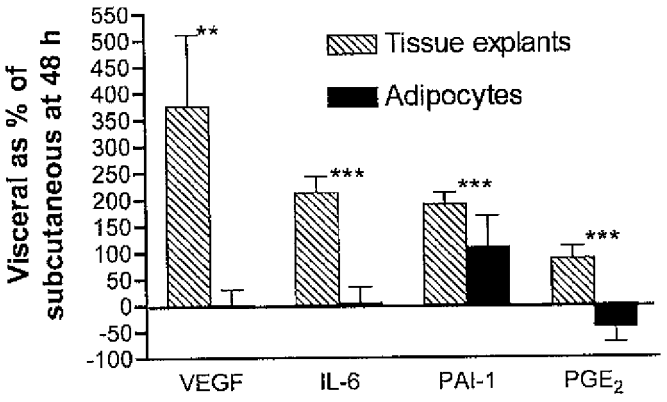

FIG. 1. Comparison of adipokine release by visceral *vs.* sc adipose tissue explants and adipocytes. The data are shown as the percentage increase ±SE of eight paired replications for adipokine release by explants of visceral adipose tissue or adipocytes derived from visceral adipose tissue over that by sc adipose tissue explants or adipocytes from the same individuals (average BMI: 32). Statistically significant differences are indicated as follows: **, $P < 0.025$; ***, $P < 0.01$.

that by tissue explants (Fig. 2). There was little difference between formation by the tissue matrix, SV cells, or adipoctyes as a percentage of that by tissue explants at 24 compared with 48 h. The one exception was an increase in leptin formation by adipocytes between 24 and 48 h and a lesser decrease over the same time period in PAI-1 formation by matrix. The total recovery of formation by the three fractions obtained by collagenase digestion was comparable to that in the experiments shown Table 6. However, there was a greater recovery of adiponectin (85% of tissue at either 24 or 48 h), a greater formation of IL-1β by SV cells, and a decrease in PAI-1 formation by adipocytes. The data in Fig. 2 indicate that measurements of release by the different cell fractions at 48 h are as reliable a way to estimate relative contribution of the different cell fractions to total release as measurements at 24 h.

In both the studies shown in Fig. 2 and Table 6, leptin was made primarily by adipocytes, whereas adiponectin was also released by the adipose tissue matrix. Lactate formation like that of PAI-1, IL-8, VEGF, PGE_2, IL-10, HGF, and IL-6 was primarily an activity seen with the adipose tissue matrix cells. TNFα was the only adipokine whose accumulation over 48 h was greater by SV cells than by the cells of the tissue matrix (Table 6).

Comparison of release by tissue vs. circulating levels of adipokines and other factors

One way to examine whether adipose tissue contributes to circulating levels of the putative adipokines is to compare their release in picomoles/kilogram over 4 h of adipose tissue with their circulating levels in picomoles/liter reported for very obese individuals (BMI ≥38). The data in Table 7 indicate that the release by adipose tissue of adipokines can be divided into several categories. Some adipokines are present in blood at very low concentrations but over 4 h are released by adipose tissue in amounts far greater than their circulating levels. These adipokines (IL-6, IL-8, PGE_2, IL-10, IL-1β, TNFα, VEGF, and HGF) are probably paracrine factors because their plasma concentrations are very low. However, the rate of release of IL-6, IL-8, and PGE_2 is so high that they could potentially contribute to circulating levels. PAI-1 is in a separate category because its circulating concentration in picomoles/liter is 24- to 12,500-fold higher than those of IL-6, IL-8, PGE_2, IL-10, IL-1β, TNFα, VEGF, or HGF. Therefore, it is more likely that PAI-1 release by adipose tissue could contribute to circulating levels of PAI-1.

Leptin and adiponectin are circulating hormones released primarily by adipose tissue, and their ratios of release divided by circulating levels are 0.13 and 0.04. If we assume that humans with a BMI of 45 have 56 kg of fat and a plasma volume of 6 liters, this gives a ratio of 0.11 for plasma volume in liters divided by fat in kilograms. Thus, over 6 h the release of leptin, if it occurred *in vivo* at rates comparable to that seen *in vitro* over 4 h, would be comparable to the blood concentration of leptin.

Total release of adipokines at a BMI of 45 compared with 32 based on release over 48 h

Another way to compare release of adipokines is to express the data as total release of adipokines by adipose tissue and adipocytes over 48 h rather than per gram of adipose

TABLE 6. Comparison of adipokine release with that of glycerol and lactate by the three fractions obtained after collagenase digestion of human adipose tissue

Released substance	Formation by SV cells (% of tissue)	Formation by tissue matrix (% of tissue)	Formation by adipocytes (% of tissue)	Formation by adipocytes (% of matrix + SV cells)
Leptin	0.1	4	69	1680
Glycerol	5	28	31	94
Adiponectin	2	29	20	64
PAI-1	3	33	9	25
Lactate	15	58	12	16
IL-8	12	53	8	12
VEGF	6	69	6	8
IL-6	6	51	4	7
IL-1β	23	78	6	6
PGE_2	14	57	5	7
IL-10	5	40	2	4
HGF	4	45	2	4
TNFα	77	34	7	6

The values for adipocytes, SV cells, and the cells of the undigested tissue matrix are expressed as percentage of the activity per gram of adipose tissue that was taken for digestion. The values were obtained by pooling data from visceral and sc adipose tissue, tissue matrix, SV cells, and adipocytes from eight individuals with a BMI of 32 and eight with a BMI of 45. The data for adipose tissue and adipocytes from visceral adipose tissue are from the experiments shown in Table 4, whereas those for sc adipose tissue and adipocytes are from Table 5.

tissue. The data in Fig. 3 are shown as release at a BMI of 45 divided by that at a BMI of 32. The data are the average of the pooled values for visceral and sc adipose tissue or adipocytes. Two major conclusions can be drawn from the data shown in Fig. 3. The first is that the release of TNFα, IL-8, and IL-10 are markedly greater in adipose tissue from women with a BMI of 45 compared with those by adipose tissue explants from women with a BMI of 32. The second is that these marked differences are not seen in adipocytes except for TNFα. There were 46 and 75% elevations in release of IL-8 and IL-1β, respectively, by adipocytes from women with a BMI of 45, but for most adipokines total release by adipocytes was reduced at a BMI of 45 compared with 32 (Fig. 3). These comparisons are approximations because we have no estimate of the relative contribution of visceral compared with sc adipose tissue to the total body fat content.

Discussion

The present studies suggest that human adipose tissue in massively obese individuals is a major site for the synthesis and release of adipokines and other factors. It was surprising that the release of PGE_2, IL-8, or IL-6 was much greater than that of adiponectin at all time periods. Although the release of PAI-1 by adipose tissue explants was smaller than that of adiponectin at short time periods, it was greater over 48 h of incubation. The very high rates of release of PGE_2, IL-8, IL-6, and PAI-1 suggest that their release by adipose tissue could theoretically contribute to the blood levels of these factors. Total IL-8 release (release per gram of tissue times the amount of fat) was 4-fold greater by adipose tissue from individuals with a BMI of 45 compared with tissue from those with a BMI of 32. However, although Bruun *et al.* (18) found that plasma levels of IL-8 were elevated in obese subjects after a 24-wk period of weight loss that reduced BMI by 15% there was actually an elevation rather than a decrease in plasma IL-8.

It is established that adiponectin and leptin are hormones released by adipose tissue to the circulation. Possibly PAI-1 is also a hormone released by adipose tissue because its circulating level is comparable to that of leptin in obese individuals and PAI-1 release by adipose tissue explants *in vitro* is greater than that of leptin. However, Yudkin *et al.* (39) found no evidence for release of PAI-1 by human sc adipose tissue based on *in vivo* measurements. Furthermore, Bastard *et al.* (40) found an increase in PAI-1 protein as well as mRNA in human sc adipose tissue after obese patients lost 5.8 kg on a very low-calorie diet. Our data are in agreement because we found a higher total PAI-1 release by adipocytes from individuals with a BMI of 32 compared with those with a BMI of 45 and no difference in the release of PAI-1 from adipose tissue *in vitro*. Alessi *et al.* (41) found that accumulation of hepatic fat was more closely correlated with plasma PAI-1 than was the amount of adipose tissue. The most likely explanation for the elevation of circulating PAI-1 in obesity is still an elevation in TNFα secretion that has been seen in obese mice (42).

The present results indicate that most of the so-called adipokines released by adipose tissue *in vitro* come from cells other than mature adipocytes. It should be noted that we incubated the adipose tissue explants with shaking for 30 min before the experiments to remove factors released during cutting the tissue as well as circulating blood cells. The cells that make most of the adipokines in washed adipose tissue explants are not macrophages or the so-called preadipocytes that are readily released from the tissue during collagenase digestion. Rather, they are the cells in the human adipose tissue matrix that are resistant to dissociation during collagenase digestion and release far more adiponectin, PAI-1, IL-8, VEGF, IL-6, IL-1β, PGE_2, IL-10, and HGF than the SV cells. TNFα is the exception because its release by SV cells was twice that by the matrix cells.

The undigested tissue matrix of human adipose tissue has been a neglected entity because digestion of rodent tissue with collagenase results in relatively little undigested material that does not pass through a 200-μm mesh filter. This is not the case with regard to human adipose tissue where there are large

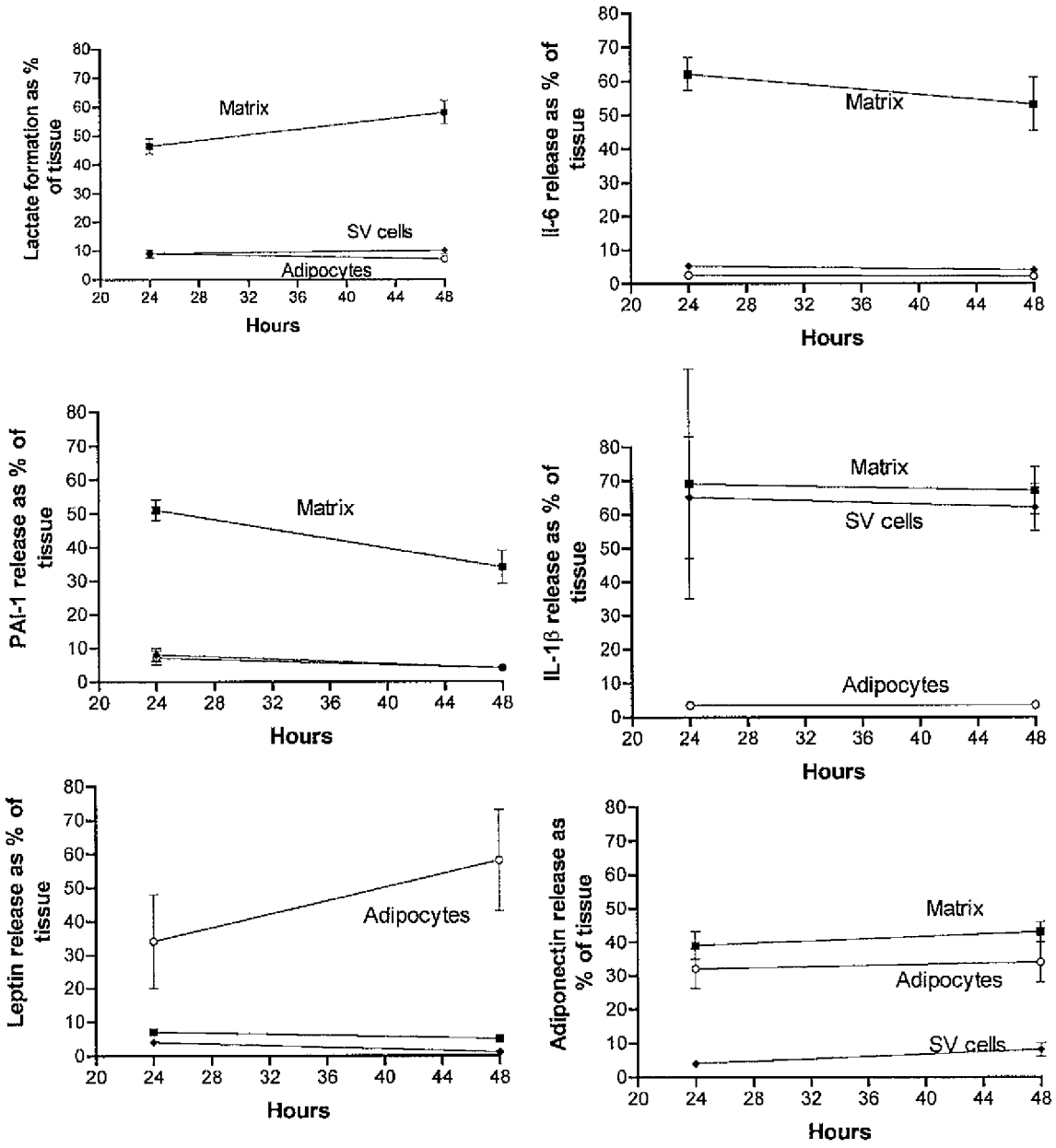

FIG. 2. Comparison of adipokine release at 24 and 48 h by the three fractions obtained after collagenase digestion of human adipose tissue. The values for adipocytes, the SV, and the undigested tissue matrix are expressed as the percentage of the activity per gram of adipose tissue that was taken for digestion from the same experiments. The values are the mean ± SEM of the data for 12 experiments (six with sc and six with visceral adipose tissue) from individuals with an average BMI of 43.

amounts of undigested matrix containing blood vessels and connective tissue as well as other cells imbedded within the matrix that are not released by collagenase digestion. Samad *et al.* (43) examined the localization of PAI-1 mRNA in murine adipose tissue and found a significant signal only in the smooth muscle cells within the vascular wall. However, after treatment of mice with endotoxin, PAI-1 mRNA was detected in SV cells and adipocytes (at a concentration 66% of that in SV cells). In human SV cells, the release of PAI-1 was about one third that by adipocytes over 48 h, and the release by adipocytes was 25% of that by the combined SV cells and the tissue matrix. This represented a greater (2- to 10-fold) contribution by adipocytes to total release of PAI-1 over 48 h than was seen with respect to release of IL-8, VEGF, IL-6, IL-1β, PGE_2, IL-10, or HGF.

It is established that abdominal obesity is associated with an increased risk of coronary heart disease as well as metabolic complications such as diabetes (44, 45). However, it is still unclear what is responsible for the greater risk of fat accumulation in the abdominal region as contrasted to overall adiposity. Studies on the regional differences in adipokine formation and release by human adipose tissue have reported that leptin release by human sc adipose tissue was greater than that of visceral adipose tissue (46, 47), whereas the reverse was seen with respect to IL-6 (7). Our data were comparable for IL-6 release by

TABLE 7. Comparison of adipokine release by pooled visceral and sc adipose tissue over 4 h with circulating levels of the same adipokines

Adipokine	4-h release by explants (pmol/kg)	Serum or plasma concentration (pmol/liter)	Ratio of release/circulating level
IL-6	14,600	0.19 (10, 15, 18)	77,000
IL-8	30,400	0.44 (18)	69,000
PGE_2	152,000	27 (36)	5,600
IL-10	194	0.13 (15)	1,490
IL-1β	18	0.07 (37)	257
TNFα	201	2 (10)	100
VEGF	22	0.66 (38)	33
HGF	746	36 (26)	21
PAI-1	1,660	875 (22,24)	2
Leptin	595	4,625 (34)	0.13
Adiponectin	6,100	173,200 (35)	0.04

Explants of visceral or sc adipose tissue were incubated for 4 h from individuals with an average BMI of 45. The values for *in vitro* release are from Table 1. Adipokine release to the medium is in picomoles per kilogram, whereas circulating levels are in picomoles per liter and are taken from the cited references.

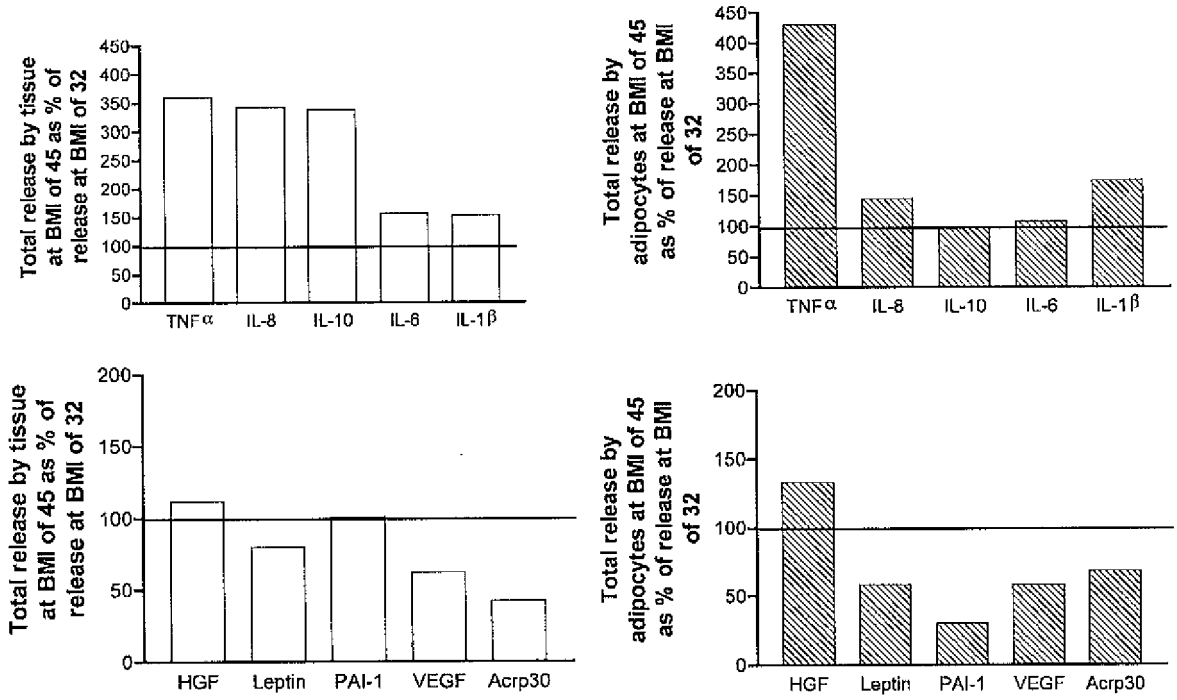

FIG. 3. Total release of adipokines at BMI of 45 compared with that at BMI of 32. The total release of adipokines was obtained by averaging the data for visceral and sc adipose tissue explants or adipocytes per gram from Tables 4 and 5. The data for release at a BMI of 45 were multiplied by 1.75 to correct for the 75% greater amount of fat (56 kg) in these individuals compared with that (32 kg) in those with a BMI of 32. Acrp30, Adiponectin.

tissue explants but not by adipocytes, where we found no differences. Leptin release by sc adipose tissue explants was also greater than that by visceral adipose tissue explants from subjects with a BMI of 45 but not by explants in those with a BMI of 32. There is controversy with regard to PAI-1 and TNFα with differences being seen in some studies but not in others as reviewed by Arner (48). TNFα release was greater by sc adipose tissue or adipocytes than by that from visceral tissue or adipocytes when tissue was obtained from humans with a BMI of 42 but not from those with a BMI of 32 in our studies. These data suggest that BMI can affect regional differences in release of adipokines.

Alessi *et al.* (49) found an enhanced release of PAI-1 by explants of visceral adipose tissue compared with that by sc adipose tissue from individuals with a mean BMI of 27. Their results were comparable to ours using tissue from individuals with an average BMI of 32 in that PAI-1 secretion was greater by visceral adipose tissue and secretion by adipocytes was about 30% of that by tissue. The greatest difference in our studies between visceral and sc adipose tissue release of adipokines was that of VEGF, which was 400% greater by visceral adipose tissue. This and the increased PGE_2 secretion by visceral adipose tissue have not been previously reported.

The increased release of IL-6, VEGF, and PGE_2 by explants but not by adipocytes of visceral adipose tissue compared with sc suggests that visceral adipose tissue has more nonfat cells that produce these factors than is the case for sc adipose tissue. The increased release of PAI-1 by visceral explants was also

seen in adipocytes, but the difference was cut in half. Bastelica *et al.* (50) suggested that PAI-1 was released by the nonfat cells of human adipose tissue rather than adipocytes and that stromal cells are more numerous in the visceral than in sc adipose tissue. The elevated blood levels of, TNFα (11, 25), IL-8 (18, 20), and IL-6 (11–19, 29) in obese individuals may reflect increased release of these adipokines by human adipose tissue because of the high levels of their release *in vitro*. This is supported by our data that their release by adipose tissue explants was greatly enhanced at a BMI of 45 compared with 32. Circulating IL-10 is elevated in obesity (15), and we found a correlation between BMI and release of IL-10 in sc but not in visceral adipose tissue. However, the release of IL-10 was far smaller than that of IL-8 or IL-6, and it appears less likely that IL-10 release by adipose tissue contributes to circulating levels of IL-10.

There are no reports of elevations of circulating levels of PGE_2, VEGF, or IL-1β in obese humans. Furthermore, our data did not indicate any increases in the release of PGE_2, VEGF, or IL-1β in adipose tissue from morbidly obese individuals with a BMI of 45 compared with their release by fat from individuals with a BMI of 32. IL-18 is another cytokine whose concentration in blood is some 100-fold higher than those of IL-6, IL-8, IL-10, or HGF and elevated in obesity (51). However, we measured the release of IL-18 by human adipose tissue explants and found that it was approximately one tenth that of IL-1β (our unpublished results). This suggests that the elevated levels of this cytokine seen in obesity do not reflect release by adipose tissue.

The release of TNFα by both adipose tissue and adipocytes over 48 h is probably a reflection of their release during the first hours of incubation because there was no net increase in their release between 4 and 48 h. It is unclear why net accumulation of TNFα decreases after 4 h of incubation as this could reflect either an inhibition of TNFα formation or enhanced rate of degradation. Our data are quite different from those of Gesta *et al.* (52), who reported a substantial up-regulation of TNFα in the adipocytes that were isolated from adipose tissue explants after 24 or 48 h of incubation in medium containing serum. However, our data are in agreement with prior reports of greater release of TNFα (27, 28) as well as greater amounts of TNFα mRNA (27) in sc adipose tissue from obese humans. Most of this TNFα release was due to nonfat cells, but there was also enhanced release by adipocytes isolated from sc adipose tissue of massively obese individuals (BMI of 45).

One problem in the interpretation of data obtained by measuring release of factors by adipose tissue and fractions derived from adipose tissue is the possibility that manipulation of the tissue may alter the rate of adipokine release. Ruan *et al.* (53) found that there was a marked up-regulation over 24 h in the level of IL-6 gene expression as well as release by adipocytes after the removal of adipose tissue from mice. In our studies the release of IL-6 during the first 4 h of incubation was comparable to the release of IL-6 per 4 h in human adipose tissue or adipocytes incubated for 48 h. In contrast, we saw a marked up-regulation of PAI-1 over 48 h in both human adipose tissue and adipocytes. The major differences in release of factors between 4 and 48 h between human adipocytes isolated by collagenase digestion and adipose tissue was a significant up-regulation over time in PGE_2 and IL-8 release and down-regulation of VEGF release in adipocytes but not in adipose tissue. The release of the other adipokines did not significantly change between 4 and 48 h of incubation or was affected to the same extent in both adipocytes and adipose tissue making it unlikely that collagenase digestion affected their rate of release over time. The possibility must be kept open, however, that rapid changes in gene expression might occur during the less than 1 h that it takes for the removal and mincing of the adipose tissue. We have done studies examining the release of IL-6 after a 2-h incubation of adipose tissue explants and found that it was slightly but not significantly greater per h than the rate per h over 48 h (our unpublished experiments). Our data suggest that if there is any effect of removal and mincing of human adipose tissue on subsequent release of IL-6 it is rapid in onset and sustained over 48 h.

In conclusion, we have shown that human adipose tissue in primary culture releases more PGE_2, IL-8, and IL-6 than of adiponectin or leptin to the medium. The release of PAI-1 and HGF was less than that of adiponectin over 4 h but greater than that of leptin. IL-10 and TNFα were released in amounts less than those of leptin, whereas VEGF and IL1-β were released in much lower amounts. Over 90% of adipokine release by adipose tissue, except for adiponectin and leptin, was due to nonfat cells. Although PAI-1 was released to the medium by adipocytes in amounts 30% of that by the tissue matrix the release of all other adipokines by adipocytes was less than 15% of that by the tissue matrix. Furthermore, the greater release of VEGF, IL-6, and PAI-1 by visceral adipose tissue as opposed to abdominal sc adipose tissue was due to the nonfat cells of the tissue. The greatly enhanced total release of TNFα, IL-8, and IL-10 over 48 h by adipose tissue from individuals with a BMI of 45 compared with a BMI of 32 is primarily due to the nonfat cells present in the adipose tissue.

Acknowledgments

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EXHIBIT C

Isolation and characterization of cells from rat adipose tissue developing into adipocytes

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Abstract To identify cells developing into adipocytes by accumulation of triglyceride, rat epididymal fat pad cells from small rats were exposed to ^{3}H-labeled chylomicron fatty acids in vivo and then liberated with collagenase. Tissue remnants were removed by filtration and mature fat cells by flotation. Aggregating cells were then removed by filtration through a 25-μm nylon screen. Further purification of cells labeled in vivo was obtained by removing floating cells from those adhering to the bottom of a culture dish. The adhering cells multiplied to a confluent monolayer when cultured in Medium 199 containing serum, glucose, insulin, and a triglyceride emulsion. The cells then gradually enlarged due to granulation of the cytoplasm by a lipid-staining material. After about 2 weeks these granules had coalesced forming mature adipocytes of typical signet-ring appearance. Free adipocytes could then be recovered from the cultures by collagenase treatment. After about 2 weeks of culture these cells had the same size (about 30 μm) as adipocytes recovered in the original collagenase preparation of the rat epididymal fat pad. They contained triglyceride lipase activity and incorporated glucose into triglycerides to the same extent as cells developed in vivo but had higher lipoprotein lipase activity. In vitro, heparin in a low concentration, prostaglandin E_1, isobutylmethylxanthine, and cholera toxin markedly promoted the development of these cells into adipocytes. This could be shown to occur almost completely indicating that this fraction of cells was homogeneous and consisted of cells with the capacity to form adipocytes. The duplication time was about 2 days and did not change with subculturing. Preadipocytes could be obtained by density gradient centrifugation, isolating triglyceride-containing cells either directly from the pad or after 3 days in culture. All of these cells developed into adipocytes as described above but did not multiply as readily. It was concluded that cells from the epididymal fat pad from small rats can be isolated in a homogenous fraction that develops in culture into cells of identical morphology and function as adipocytes formed in vivo. The differentiation of these cells into adipocytes may be manipulated in vitro.

Supplementary key words preadipocytes · collagenase

Research utilizing histological techniques for examination of adipose tissue in situ has not been able to identify the origin of the mature monovacuolar adipocyte in adipose tissue with either conventional (1) or electron microscopy (2). Suggestions have been put forward that adipocytes either originate from fibroblasts (1) or from specialized cells different from fibroblasts (2). However, conclusive evidence on this point seems to require longitudinal follow-up of the development of isolated, defined cells to ascertain their identity.

Hollenberg and Vost (3) have used DNA labeling in vivo to follow the formation of new fat cells. With this technique the influence of age and other factors on adipocyte multiplication has been reported (3, 4).

Recent examinations of adipose tissue cellularity have revealed associations between fat cell size and number on the one hand and clinical conditions such as obesity, hyperlipidemia, and diabetes mellitus on the other (5–7). The hyperplasia of adipose tissue has attracted attention particularly in the pathogenesis and treatment (8) of obesity. In order to define these problems further, knowledge is needed about adipocyte precursors in adipose tissue and their multiplication and development.

A first step towards a better elucidation of the problems mentioned above would be the separation of fat cell precursors from adipose tissue for subsequent characterization. The present work describes such isolation procedures resulting in a homogeneous fraction of cells that develops into mature adipocytes upon subsequent culture. Preliminary reports of this work have been presented previously (9, 10).

EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats, 19–25 days of age, weighing 40–65 g were mainly utilized. They were fed ordinary rat chow containing by weight 5% fat, 55% carbohydrate, and 22.5% protein plus

minerals and vitamins (EWOS, Södertälje, Sweden) and water ad libitum. Boiled potatoes were also given to the smallest rats. The temperature was not allowed to fall below 25°C and the rats were housed at least five in each cage. A few rats weighing 275–300 g were also utilized. These were housed in single cages and were given rat chow and water ad libitum.

Rats were killed by exsanguination under diethyl ether anesthesia. The epididymal fat pads and testes were removed *in toto* under sterile conditions and placed in 0.9% saline in a Petri dish for removal of the epididymal fat pad from the border of the epididymis. The blood vessels were then cut off where they entered the fat pad, opened as far as possible, and remaining blood was washed away.

Labeling of epididymal fat pad cells in vivo

About 15 hr before removal of the fat pads the rats were given by a stomach tube, under light ether anesthesia, an emulsion of 0.2 ml of corn oil, and 0.2 ml of absolute ethanol in which was dissolved 5 mCi of [U-^{3}H]palmitic acid (NET-043, New England Nuclear, Boston, MA). After that the drinking water was replaced with 10% sucrose over night.

Liberation of cells

Two fat pads from the small rats or one from the larger rats were incubated in a 50-ml siliconized Erlenmeyer flask containing 10 ml of a solution with final concentrations of 0.1 M HEPES (hydroxyethylpiperazine-ethane-sulfonic acid, Sigma, St. Louis, MO) buffer, 0.12 M NaCl, 0.05 M KCl, 0.001 M $CaCl_2$, 0.005 M glucose, and 1.5% (w/v) bovine serum albumin (Bovine serum albumin Fraction V, batch WB 1370, Armour, Eastbourne, England). This solution contained 0.2% (w/v) collagenase (Worthington, Freehold, NJ, batch CLS 46D112X). The gas phase was air; the pH was 7.4; and the temperature was 37°C. Incubation time was 30 min if not noted otherwise. Incubations were performed in a water bath with rotating flasks (120 rpm).

After incubation the tissue remnants were removed by filtration through a nylon screen with a pore size of 250 μm into a siliconized test tube. The fat cells were then allowed to float to the surface for 15 min whereafter the infranatant was aspirated through a siliconized injection needle.

The infranatant was then filtered through a nylon screen with a pore size of 25 μm to remove cell aggregates. The cells passing the filter were pelleted by centrifugation and then resuspended in the appropriate buffer or medium for further processing.

Cell culture

Cells were cultured under sterile conditions essentially as previously described (11) in Leighton tubes, Petri dishes, or in larger tissue culture flasks (Costar, Cambridge, MA) at 37°C in Medium 199 (Statens Bakteriologiska Laboratorium, Stockholm, Sweden) supplemented with 20% freshly obtained human serum, 5 mM glucose, 40 mU/ml insulin (Vitrum, Stockholm, Sweden), 0.5 (w/v) of triolein in a stable emulsion with lecithin (Intralipid, Vitrum, Stockholm, Sweden), and 0.1 mg/ml of sodium cefalothin (Keflin, Eli Lilly, Indianapolis, IN). Culture media were changed at least every second day. When needed for inspection or photographic registrations, media without lipid were temporarily utilized. Cells were removed by incubation for 10 min at 37°C with 0.125% (w/v) trypsin (Sigma, Type I, St. Louis, MO) in 0.15 M phosphate buffer, pH 7.0.

Heparin was obtained from Vitrum, Stockholm, Sweden, isobutylmethylxanthine from Aldridge, Milwaukee, WI, theophylline from Sigma, St. Louis, MO, epinephrine from Astra, Södertälje, Sweden. Prostaglandin E_1 was a gift from Professor Sune Bergström, Karolinska Institutet, Stockholm, Sweden. Purified cholera toxin was from Dr. Jan Holmgren, University of Göteborg, Sweden.

Density gradient centrifugation

The density gradient system with colloidal silica and polyvinylpyrrolidone described by Pertoft and Laurent (12) was utilized; however, Medium 199 was used instead of sucrose. Osmotic pressure was 300 mOsm, pH 7.4. Linear gradients were made utilizing the device described by Britten and Roberts (13) in 50-ml test tubes 10 cm in height. Cells were pipetted to the top of the tube in 5 ml of Medium 199. Centrifugations were then performed at 800 *g* for 40 min at room temperature in a swing-out head. Densities were determined in kerosene–carbon tetrachloride columns (14) utilizing sucrose solutions of known densities as standards. Fractions were obtained from the top of the gradient and the cells were washed and pelleted. Radioactivity was counted in chloroform–methanol 2:1 extracts of the cells.

Other methods

Cell size measurements were performed by an eyepiece in the ocular of the microscope. Determinations of cell number were performed in a blood cell counting chamber. Staining for cellular contents of lipid was performed with Oil Red O.

Lipoprotein lipase activity was determined according to Nilsson-Ehle, Tornqvist, and Belfrage (15).

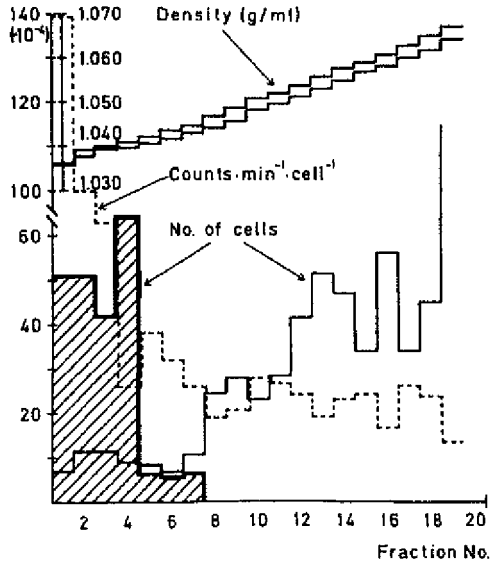

Fig. 1. Results of density gradient centrifugations of heavy cells obtained from the epididymal fat pads by collagenase incubation and removal of aggregating cells by filtration. Cells labeled in vivo with [U-^{3}H]palmitic acid. Shaded area is cell distribution after 3 days in culture medium. (Radioactivity was not measured in these cells.)

Glycerol release and incorporation of [1-^{14}C]glucose (NEC-043, New England Nuclear, Boston, MA) into tissue lipids were performed as described previously (16).

DNA was determined according to Kissane and Robins (17) after initial delipidization in acetone and diethyl ether (15) with subsequent homogenization in glass homogenizers that could be centrifuged. Triglyceride concentration was determined colorimetrically as triglyceride-glycerol after hydrolysis according to Carlson (18).

Statistical calculations were performed with Student's *t* test.

RESULTS

Initial purification of lipid-accumulating cells

Labeled fatty acids were administered to the adipose tissue donor rat in order to identify cells other than mature fat cells taking up lipid in vivo. As will be seen below, two problems arose in the subsequent isolation of these cells. One problem was that the mature fat cells took up the majority of the label, and therefore the appearance of fat cell ghosts during the preparation had to be avoided because of the risk that these ghosts contained some remaining highly labeled triglyceride. The other problem was the aggregation of nonfloating cells during the subsequent separation procedures.

In order to prevent the first problem it was necessary to approach the collagenase procedure as cautiously as possible. Utilizing the method described in the Experimental Procedure section, the majority of the fat cells and nonfloating cells were already liberated after incubation for 30 min. Free triglyceride, indicating the formation of fat cell ghosts, was seen only at 60 min.

Aggregation of the nonfloating cells visible by inspection or in the microscope was found after 15 min of incubation, but tended to increase at 45 and 60 min and, at the latter time points, DNA:ase was needed to diminish aggregation. This finding as well as the free-floating fat after collagenase treatment for 60 min indicated a breakdown of cell integrity. Thirty minutes of collagenase treatment was therefore chosen for further work.

The procedure selected for the initial steps of cell preparation was thus the following. After collagenase treatment for 30 min tissue remnants were removed by a 250-μm filter. Adipocytes were then allowed to float to the surface and the remaining medium was aspirated through a siliconized needle into a plastic syringe. Thereafter cell aggregates were removed by a 25-μm nylon screen.

The cells of the filtrate could be further separated by density gradient centrifugation. **Fig. 1** shows that there was a peak activity of incorporated fatty acids of the cells in the lighter part of the gradient, although activity was found in cells all over the gradient.

When the cells obtained after filtration through the 25-μm screen were placed in a culture flask, the cells adhering to the bottom contained about twice as much radioactivity per cell as those remaining

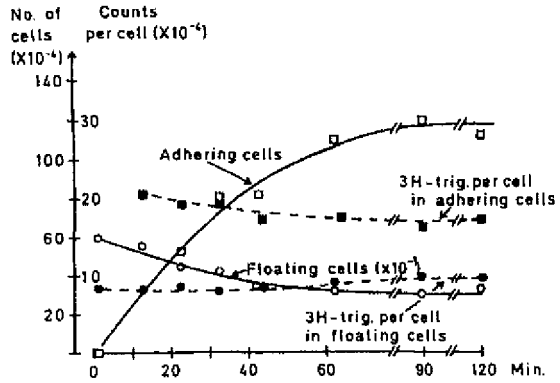

Fig. 2. Adherence of cells to the bottom of a culture flask and their triglyceride label from in vivo labeling. Number of floating cells $\times 10^{-1}$.

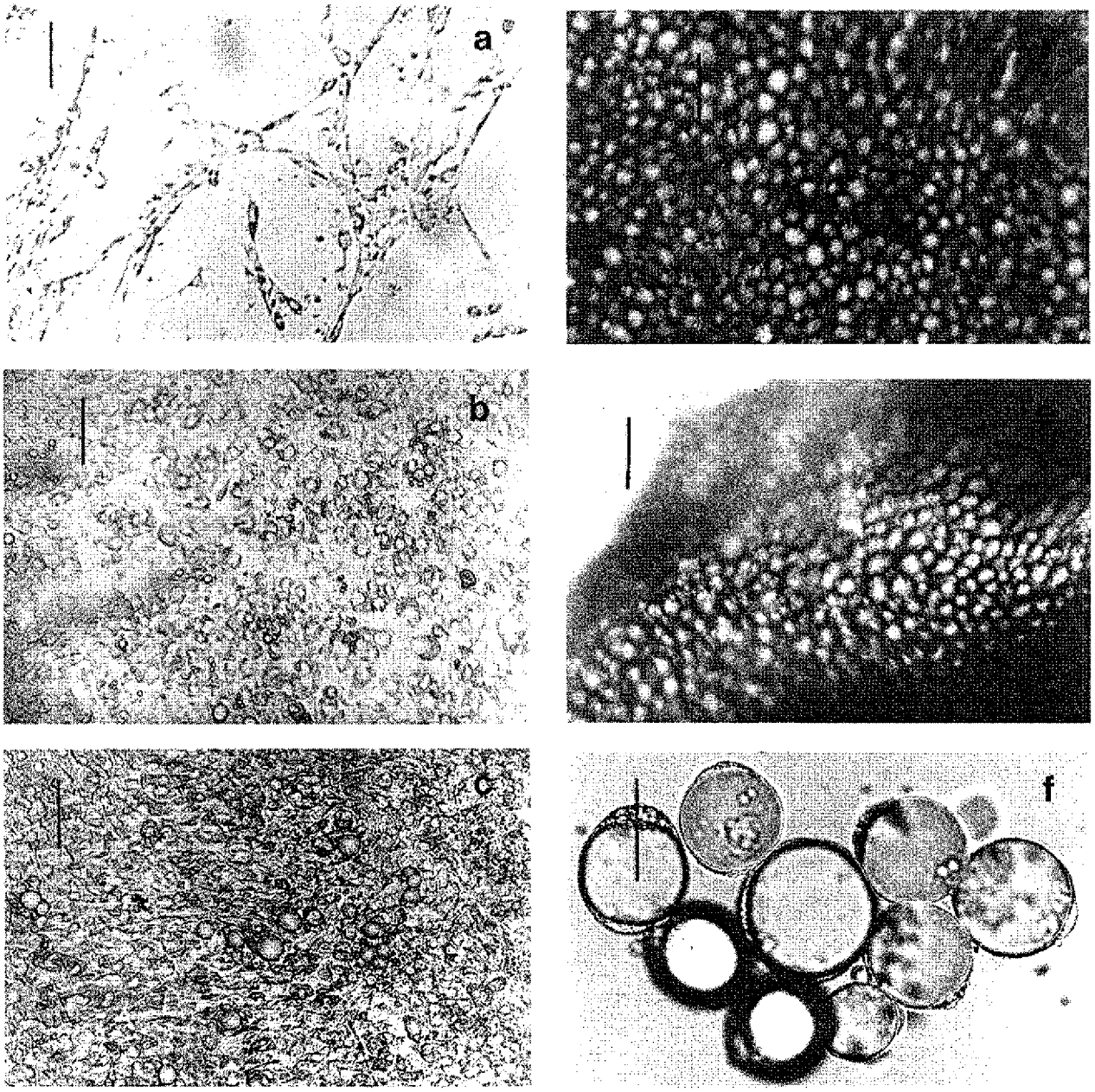

Fig. 3. Adipocyte development in vitro in culture. *a*) Cells in the multiplication state (2 days of culture). Black bar; 50 μm. *b*) Confluent monolayer with enlarged cytoplasm containing multiple granules (4 days of culture). Black bar, 50 μm. *c*) Granules coalescing (6 days of culture). Black bar, 50 μm. *d*) Univacuolar adipocytes on bottom of culture tube (14 days of culture). Black bar, 50 μm. *e*) Floating collection of cells containing univacuolar adipocytes (18 days of culture). Black bar, 50 μm. *f*) Adipocytes isolated from cell collection in Fig. 5*e*. Black bar, 30 μm.

in the culture medium (**Fig. 2**). After 1 hr about one-third of the total number of cells added to the culture flask adhered to the bottom.

Cells in culture

When the cells adhering to the bottom of the culture flask were cultured in the supplemented Medium 199 the usual development was that seen in **Fig. 3**. After attachment to the bottom of the culture tube the cells assumed an elongated, thin shape and multiplied in monolayers, first in a web-like pattern (Fig. 3*a*). After a few days, when a confluent monolayer had formed, the cytoplasm became enlarged and now contained numerous granules of lipid-staining

TABLE 1. Triglyceride accumulation of cells in culture

Exp No.	Rat Weight (g)	Addition to Medium	Percent of Control
1	48–52[a]	0	100 (3)
	48–63	0	178 (3)
	278–315	0	22 (1)
2	46–67	0	100 (5)
	46–67	heparin (5 μg/ml)	230 (5)
	46–67	heparin (5 μg/ml) plus calcium (20 mM)	235 (5)
	46–67	calcium (20 mM)	9 (5)
	46–67	heparin (2500 μg/ml)	12 (5)
3	45–64	0	100 (3)
	45–64	epinephrine (10 μg/l)	110 (3)
	45–64	PGE_1 (15 μg/ml)	210 (3)
	45–64	theophylline (1.0 mM)	92 (3)
	45–64	theophylline (10.0 mM)	24 (3)
	45–64	IBMX (0.5 mM)	200 (3)
	45–64	IBMX (1.0 mM)	170 (3)
4	46–63	0	100 (3)
	46–63	cholera toxin (0.01 μg/ml)	222 (3)
	46–63	cholera toxin (1.0 μg/ml)	230 (3)

[a] Rats taken directly after delivery to the laboratory. Other rats were acclimatized for 3 days or more.

Cultures in Medium 199, supplemented with serum, glucose, insulin, and triglyceride (see Methods). IBMX, isobutylmethylxanthine; PGE_1, prostaglandin E_1.

In each experiment density fractions <1.040 g/ml were isolated, and cells in these fractions were counted. Numbers of floating adipocytes, removed before centrifugation (see text), were added. Results are expressed as percent of cells in this fraction of the control group. Percentage of cells in the fraction d < 1.040 g/ml was 37, 21, 44, and 30% for experiment no. 1, 2, 3, and 4, respectively. Indicated substances were present during the whole culture period, which was 6–9 days.

material (Fig. 3*b*). These droplets coalesced in the following days into larger granules, eventually producing one large droplet (Fig. 3*c*). After about 2 weeks the picture was frequently dominated by univacuolar cells (Fig. 3*d*). At this stage the whole mass of cells floated up from the attachment to the bottom into the culture medium (Fig. 3*e*). These floating collections of cells could be recovered and incubated with collagenase, whereby fat cells could be isolated in the same way as in the first incubation of the fat pad (Fig. 3*f*).

Effects of different factors on adipocyte formation

There was a variation in the extent to which the cells developed into adipocytes in this fraction of cells. In some preparations only a few adipocytes developed while in others the adipocytes dominated. It was noted that this variation was dependent on, for example, the condition of the rat donor. Therefore, preliminary attempts to quantitate the effect of different factors on the development of these cells into adipocytes were made. Such a quantitation was obtained by determination of the density of cells after development to a stage where a confluent monolayer of cells had formed and accumulated lipid but had not yet detached from the bottom, viz., after about 1 week in culture. At this stage the cells were removed with trypsin. Cells floating after having been left in the flask for 15 min on the laboratory bench were removed and counted. The infranatant was placed on a density gradient with densities increasing step-wise from 1.020 to 1.040 g/ml, from 1.040 to 1.055 g/ml, and from 1.055 to 1.070 g/ml. After centrifugation to equilibrium, cells in these density bands were collected, pelleted, and counted. The cells initially removed by floating were added to the d < 1.040 g/ml fraction (**Table 1**). Experiment 1 showed that the degree of acclimatization of the donor rats appeared to have an effect, because cells accumulating triglyceride were more abundant in rats taken 3 days after the arrival to the laboratory than in rats examined immediately after arrival. Larger rats showed much lower values. Experiment 2 indicated the marked effect of heparin in a low dose with or without calcium, while calcium alone or a high concentration of heparin were markedly inhibitory. In experiment 3 different agents modifying lipolysis were tested. Only a small, probably insignificant, increase in the epinephrine-containing flasks was found. There was a marked increase with prostaglandin E_1 and also with the phosphodiesterase inhibitor isobutylmethylxanthine, but not with theophylline. The higher concentration of theophylline actually inhibited the formation of light cells. Experiment 4 showed the marked effect of cholera toxin, a potent adenylate cyclase stimulator (19).

Fig. 4 shows the distribution of cells in the

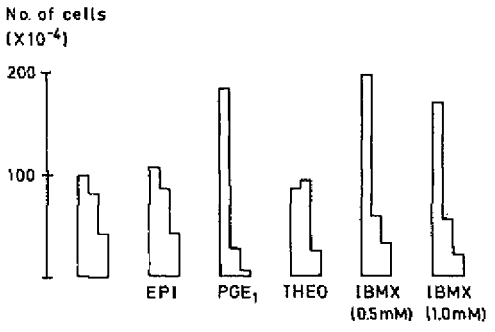

Fig. 4. Effects of different agents on the distribution of cells in the density gradient after one week in culture. Left column, density <1.040 g/ml. Middle column, density 1.040–1.055 g/ml. Right column, density 1.055–1.070 g/ml. Epi., epinephrine (10 μg/ml). PGE_1, prostaglandin E_1 (15 μg/ml). THEO, theophylline (1.0 mM). IBMX, isobutylmethylxanthine. Additions present during the whole period of culture. Means of three flasks for each condition.

gradients of experiment 3 in more detail. In this preparation about 44% of all cells developed spontaneously into cells with a density < 1.040 g/ml. The distribution pattern is fairly similar in the experiments with no addition to the medium and in those containing epinephrine or theophylline. The isobutylmethylxanthine experiments show clearly a distribution change towards lighter, triglyceride-containing cells and this is particularly the case with prostaglandin E_1 where actually 85% of all cells had developed into cells with the lightest density. With a low dose of heparin without calcium, or with heparin and calcium (experiment 2, Table 1), and in flasks containing cholera toxin (experiment 4) 83, 85, and 91%, respectively, of the cells developed into the lightest cells (not shown). The agents stimulating formation of cells in the lightest fraction apparently did not interfere with the rate of cell multiplication because the state of confluence was noted to occur at the same time in all flasks; as exemplified in Fig. 4, the number of cells in each experiment was similar.

Isolation and subsequent culture of cells with some accumulation of triglyceride

Cells cultured for 3 days, leading to accumulation of triglyceride to approximately the same degree as seen in Fig. 3*c*, could be removed with trypsin and separated by density gradient centrifugation (Fig. 1). The majority of cells in this experiment had densities less than 1.030 g/ml and these cells were harvested and cultured. In culture these cells multiplied less readily than those obtained directly after the filtration procedures but, on the other hand, they all seemed to develop into adipocytes. The same characteristics were found for cells obtained in the same density region of the gradient after direct centrifugation of the filtrate, cells also accumulating much triglyceride label in vivo (Fig. 1).

Fat cell ghosts, prepared by exposing washed fat cells to a hypotonic solution, did not grow in the culture system. The aggregating cells or fibroblasts grown from the tail skin of rats weighing about 300 g accumulated no visible lipid or only sparse granules of lipid-staining material.

Functional characteristics of the lipid-accumulating cells

Fig. 5 shows some functional characteristics of cells analyzed during the preadipocyte phase and mature monovacuolar adipocytes developed in vivo or in vitro. Glycerol release was more pronounced in adipocytes than in preadipocytes. There was a lipolytic response to norepinephrine in all cells. With respect to glucose incorporation into triglycerides, the mature adipocytes also showed higher activities than the preadipocytes. On the other hand, the lipoprotein lipase activity was similar for the preadipocytes and for the adipocytes developed in vivo, while it was higher for adipocytes developed in vitro. Cells in the multiplying state in nonconfluent cultures without visible granules showed much less lipase

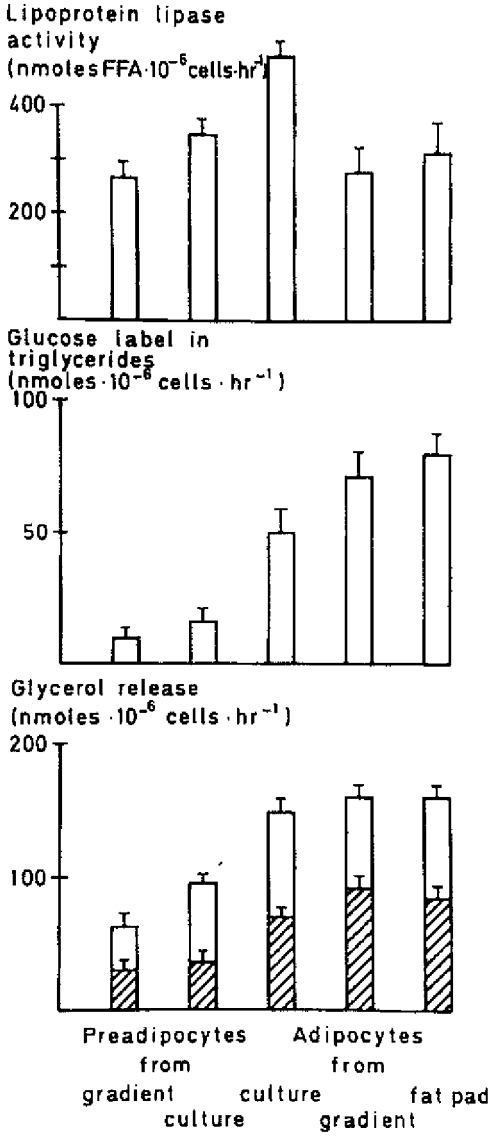

Fig. 5. Lipoprotein lipase activity, glucose incorporation into triglycerides and glycerol release (basal, striped; after 10 μg/ml norepinephrine, open) of preadipocytes obtained from top of gradient (Cf. Fig. 1) (first column), after 3 days of culture (Cf. Fig. 3 *b*) (second column), and in adipocytes after 14 days of culture (Cf. Fig. 3*f*) (third column), in adipocytes from the fat pad after 40 min exposure of gradient solution (fourth column), and in adipocytes directly from the rat fat pad after collagenase liberation (fifth column). Fat cell sizes 30 ± 4, 34 ± 5, 34 ± 5 (means ± SD) μm in columns 3, 4, and 5, respectively. Means ± SEM; n, 5–7.

and triglyceride synthesizing activities than the cells in **Fig. 5** (not shown).

The duplication time of the cells obtained by the filtration procedure averaged about 48 hr. This was apparently not changed after as many as ten subcultures, nor was the homogeneous character of the fraction changed visually.

DISCUSSION

The present work demonstrates the presence of cells in rat adipose tissue that develop in vitro into cells of identical morphology and function as mature adipocytes. This is then in agreement with previous work in vivo utilizing techniques for labeling DNA of cells subsequently developing into mature adipocytes (3).

In order to facilitate the purification of cells developing into adipocytes the donor rats were fed labeled fatty acids together with a triglyceride. This mixture should be transformed to labeled glycerides entering the blood stream as chylomicra and taken up in tissues, particularly adipose tissue, because the rats were also given a sucrose solution (20). It seems physiologically reasonable to assume that the labeled triglyceride was taken up primarily not only in adipocytes but also in their precursors rather than in other cells such as fibroblasts, blood cells, and so on. This assumption is supported by the fact that fractions of cells containing much label also developed into adipocytes. Also in agreement with this possibility is the fact that the light, highly labeled fraction obtained from the density gradient (Fig. 1) apparently developed into adipocytes totally without further purification. It is highly unlikely that adipocytes growing from this fraction originated from fat cell ghosts because of the precautions taken in the procedure to liberate the cells, and because of the fact that ghosts prepared by exposure to a hypotonic environment did not grow or multiply.

An important question is whether the isolated cells are a homogeneous population. As far as the more differentiated cells from the density gradient centrifugations are concerned, it seems probable that these are homogeneous because they were isolated on the basis of their characteristic low density due to their high triglyceride contents. They all seemed to develop into adipocytes. The reason for their decreased tendency to multiply might be characteristic for this degree of development.

The less differentiated fraction of cells obtained by the filtration procedures is no doubt less homogeneous. The heterogeneity could be due either to a variation in differentiation of the fat cell precursors or to the presence of cells other than fat cell precursors, such as endothelial cells. The latter alternative seems to be a minor problem because when potent agents were added to enhance the formation of adipocytes (heparin, prostaglandin E_1 or cholera toxin) essentially all cells developed into adipocytes. Furthermore, the cells seemed to be visually homogeneous in several subcultures with no tendencies for overgrowth of other cell types, and with an apparently equal duplication time. It cannot be decided from the present results whether the cells in the fraction obtained from the filtration procedures are a homogeneous population or whether the homogeneity of the fraction is obtained by the overgrowth of the cells that accumulate triglyceride. Such an overgrowth is presumably enhanced by the purification procedures and the culture conditons.

The heterogeneity of the fraction of cells obtained by filtration is probably primarily caused by a difference in the development of these cells. Fig. 3 shows the characteristic development with a stage of multiplication (Fig. 3*a*) and a subsequent development into mature adipocytes (Fig. 3*d*). This development was not uniform in the culture flask and not even within the groups of cells where this development was observed. Within one group of cells the central cells, which presumably were no longer multiplying because of contact inhibition, usually started to accumulate lipid, while cells at the edges were apparently still multiplying. This resulted in a heterogeneous picture within each group of lipid-accumulating cells from mature adipocytes to cells just beginning to accumulate lipid.

The possibilities of manipulating the development of the cells into adipocytes were examined. The density borders chosen here in the density gradient separations were fairly arbitrary, but all cells with a density of less than 1.040 g/ml have repeatedly been found to develop into adipocytes. This does not exclude the possibility that cells from the higher density fractions could develop into adipocytes as well.

The results of Table 1, experiment 1, indicate that the size of the rat as well as acclimatizing factors, perhaps including feeding, play a role in this development. This is in agreement with findings in vivo utilizing the DNA-labeling method (3, 4). The results of Table 1 also suggest that the cell development can be influenced by in vitro factors. The experiments with heparin and calcium indicate that factors promoting fat cell development might be similar to factors stimulating lipoprotein lipase (20). Other experiments suggested that formation of mature adipo-

cytes would be promoted by prostaglandin E_1, IBMX, and cholera toxin. In a recent report (21) the conversion of 3T3 fibroblasts into adipocytes did not seem to be dependent on cyclic AMP. These preliminary experiments in the present work allow no conclusions as far as the details of the mechanism for the cell differentiation to adipocytes is concerned. They show clearly, however, the marked possibilities directing the cell toward adipocyte formation. These experiments also show that the cells of the fraction obtained by the separation procedures are or become a homogeneous population; when the cells do not spontaneously develop into adipocytes or their precursors, essentially all the cells can be made to develop into adipocytes.

When a sufficient number of mature adipocytes had developed in the culture tubes the adhesion to the bottom diminished and eventually the flotation tendencies of the fat-filled cells also peeled off cells at earlier stages of development, resulting in the fact that the whole collection of cells left the bottom and floated in the medium. Presumably this was due to the diminished contact with the bottom of the culture tube when the cells were beginning to assume a spherical form and, at the same time, the tendency to float was increased because of the decreasing density of the cells.

A recent report by Van, Bayliss, and Roncari (22) described the characteristics of cells obtained from adult human omentum. These cells collected lipid and had the functional characteristics of mature fat cells emptied of most of their triglyceride; they were different in this respect from skin fibroblasts. A purification of the original cell mixture was apparently not necessary to obtain these cells. The subsequent development to mature adipocytes was never demonstrated, and the question of whether or not these cells represent early stages of adipocytes can therefore not be considered settled.

Green and Meuth (23) have recently described a fraction of cells (3T3-L1), isolated from mouse embryos, and some of these cells accumulate triglyceride and eventually form cells of identical morphology as mature fat cells. Although a functional comparison cannot be performed because of lack of congruent data, these cells seem to have the same appearance and growth characteristics, including the heterogeneous rate of triglyceride filling, as the cells of the fraction obtained by the filtration procedures presented here. The tendency for formation of lipid-accumulating cells seems to be more pronounced for the cells isolated in the present work. This may well be due to the fact that these cells are more highly differentiated because they were isolated after indication of triglyceride uptake in vivo, a function presumably requiring differentiation toward lipid accumulation, and because they were isolated from a defined adipose tissue. Dardick et al. (24) recently and Poznanski, Waheed, and Van (25) previously have demonstrated the presence of lipid granules in cells obtained from human adipose tissue. Similar granules were also found, although less abundantly, in fibroblasts, demonstrating the lack of specificity of this criterion. No functional characteristics were studied, and the fact that these cells did not develop into mature adipocytes of typical appearance and function prevents any conclusions about their identity.

The present study shows conclusively that cells can be isolated from the epididymal fat pad of small rats in a homogenous fraction forming in culture in a substrate-rich medium cells with the typical appearance and identical function of mature adipocytes. These cells may thus be labeled preadipocytes or adipoblasts, since essentially they can be made to develop completely by different means in vivo and in vitro into adipocytes.

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EXHIBIT D

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REGULAR ARTICLE

Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies

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Abstract The stromal vascular fraction (SVF) of adipose tissue contains an abundant population of multipotent adipose-tissue-derived stem cells (ASCs) that possess the capacity to differentiate into cells of the mesodermal lineage in vitro. For cell-based therapies, an advantageous approach would be to harvest these SVF cells and give them back to the patient within a single surgical procedure, thereby avoiding lengthy and costly in vitro culturing steps. However, this requires SVF-isolates to contain sufficient ASCs capable of differentiating into the desired cell lineage. We have investigated whether the yield and function of ASCs are affected by the anatomical sites most frequently used for harvesting adipose tissue: the abdomen and hip/thigh region. The frequency of ASCs in the SVF of adipose tissue from the abdomen and hip/thigh region was determined in limiting dilution and colony-forming unit (CFU) assays. The capacity of these ASCs to differentiate into the chondrogenic and osteogenic pathways was investigated by quantitative real-time polymerase chain reaction and (immuno)histochemistry. A significant difference (P=0.0009) was seen in ASC frequency but not in the absolute number of nucleated cells between adipose tissue harvested from the abdomen (5.1±1.1%, mean±SEM) and hip/thigh region (1.2±0.7%). However, within the CFUs derived from both tissues, the frequency of CFUs having osteogenic differentiation potential was the same. When cultured, homogeneous cell populations were obtained with similar growth kinetics and phenotype. No differences were detected in differentiation capacity between ASCs from both tissue-harvesting sites. We conclude that the yield of ASCs, but not the total amount of nucleated cells per volume or the ASC proliferation and differentiation capacities, are dependent on the tissue-harvesting site. The abdomen seems to be preferable to the hip/thigh region for harvesting adipose tissue, in particular when considering SVF cells for stem-cell-based therapies in one-step surgical procedures for skeletal tissue engineering.

Keywords Adipose-tissue-derived stem cells · Chondrogenic differentiation · Osteogenic differentiation · Colony-forming unit assay · Stem-cell yield · Tissue-harvesting site · Human

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Introduction

Tissue engineering is an emerging field in modern medicine. Therapies involve the combination of cells and scaffold materials that can be loaded with bioactive factors, ideally resulting in the regeneration or replacement of lost or

damaged tissues and organs. Multiple cell sources have been investigated for their possible applicability in tissue engineering. Embryonal stem cells are the most potent stem cells; however, their use is controversial and has mayor ethical considerations (Dresser 2001). Mesenchymal stem cells (MSCs) can be obtained from the adult and are widely used because of their differentiation potential. In addition to bone marrow, periosteum (Nakahara et al. 1991), muscle (Asakura et al. 2001), and adipose tissue (Zuk et al. 2002) also appear to be sources of MSCs.

Subcutaneous adipose tissue is a particularly attractive reservoir of progenitor cells, because it is easily accessible, abundant, and self-replenishing. It is derived from the mesodermal germ layer and contains a supportive stromal vascular fraction (SVF) that can be readily isolated (Gronthos et al. 2003; Zuk et al. 2001). This SVF from adipose tissue consists of a heterogeneous mixture of cells, including endothelial cells, smooth muscle cells, pericytes, leukocytes, mast cells, and pre-adipocytes (Oedayrajsingh-Varma et al. 2006; Peterson et al. 2005; Prunet-Marcassus et al. 2005). In addition to these cells, the SVF contains an abundant population of multipotent adipose-tissue-derived stem cells (ASCs) that possess the capacity to differentiate into cells of mesodermal origin in vitro, e.g., adipocytes, chondrocytes, osteoblasts, and (cardio)myocytes (Erickson et al. 2002; Guilak et al. 2004; Halvorsen et al. 2001; Hattori et al. 2004; Planat-Benard et al. 2004; Rangappa et al. 2003; Zuk et al. 2001). Because of these favorable characteristics, interest has been growing in the application of ASCs for cell-based therapies such as tissue engineering.

For clinical practice, an advantageous approach would be to harvest ASCs and immediately give them back to the patient within the same operation, the so called "one-step surgical procedure" (Helder et al. 2007). This overcomes long-lasting culture expansion of ASCs on the one hand, but necessitates the use of the SVF of adipose tissue on the other hand, since fast selection procedures for stem cells in the SVF are not yet available. Therefore, the success of this procedure requires SVF-isolates to contain sufficient ASCs capable of differentiating into the desired cell lineage. In view of this, we have previously investigated the effect of three different surgical procedures for the harvesting of adipose tissue, i.e., resection, tumescent or conventional liposuction, and ultrasound-assisted liposuction, on the yield and function of the stem cells. We have demonstrated that the SVF isolates from adipose tissue harvested by ultrasound-assisted liposuction contain fewer stem cells, and that the stem cells have a longer population doubling time, leading us to conclude that resection and tumescent liposuction are preferable to ultrasound-assisted liposuction for harvesting adipose tissue, if the cells are to be used for tissue-engineering purposes (Oedayrajsingh-Varma et al. 2006).

In the present study, we have investigated whether the yield and functional characteristics of ASCs in the SVF are affected by the most frequently used adipose-tissue-harvesting sites. We have previously demonstrated that the yield of nucleated cells in the SVF of the adipose tissue from these different tissue-harvesting sites is similar (Oedayrajsingh-Varma et al. 2006). In the current study, the frequency of ASCs in the SVF cell isolates has been determined by using limiting dilution and colony-forming unit (CFU) assays. In addition, we have investigated the frequency of CFUs showing an osteogenic differentiation capacity. SVF cells have been subsequently cultured in order to obtain homogeneous cell populations and to acquire sufficient cells to determine their chondrogenic differentiation potential in micromass cultures. Homogeneity has been checked by determining the growth kinetics and phenotypic characteristics of the ASCs. To verify the maintenance of multidifferentiation potential, osteogenic and chondrogenic induction has been assessed in these homogeneous ASC cultures.

Materials and methods

Donors

Samples of human subcutaneous adipose tissue were obtained as waste material after elective tumescent liposuction or resection and donated after informed consent from healthy donors operated on at the Departments of Plastic Surgery of two clinics in Amsterdam, The Netherlands. Adipose tissue was taken from the abdomen ($n=12$) and the hip/thigh region ($n=10$) during cosmetic surgery; 22 female donors were included in this study. The average age (mean age 40, range 24–62 years) and body mass index (BMI; mean BMI 25.5; range 22.2–29.6 kg/m^2) were similar for both groups (Table 1).

Cell isolation and storage

Isolation of the SVF from adipose tissue was performed as previously described (Oedayrajsingh-Varma et al. 2006). The isolation protocol included a Ficoll density centrifugation step to remove contaminating erythrocytes. After isolation, 4×10^6 SVF cells were resuspended in a mixture (1:1) of Dulbecco's modified Eagle's medium (DMEM) and cryoprotective medium (Freezing Medium, BioWhittaker, Cambrex, Verviers, Belgium), frozen under "controlled rate" conditions in a Kryosave (HCI Cryogenics, Hedel, The Netherlands), and stored in the vapor phase of liquid nitrogen according to standard practice at the Department of Pathology of the VU University Medical Center and following the guidelines of current Good Manufacturing Practice.

Table 1 Population characteristics (*BMI* body mass index, *THS* tissue-harvesting site, *ASC* adipose-derived stem cell, *Res* resection, *T-LS* tumescent liposuction, *ND* not determined)

Donor		Age (years)	BMI (kg/m^2)	THS	Procedure	ASC yield (%)
1	02-0001	62	22.6	Abdomen	Res	9.20
2	03-0007	26	24.4	Abdomen	T-LS	8.50
3	03-0018	42	23.9	Abdomen	Res	12.50
4	03-0021	57	29.6	Abdomen	Res	4.40
5	04-0003	37	ND	Abdomen	T-LS	2.20
6	04-0013	36	28.1	Abdomen	Res	2.20
7	04-0015	37	26.9	Abdomen	Res	0.93
8	05-0004	39	ND	Abdomen	T-LS	1.50
9	05-0007	42	28.3	Abdomen	T-LS	8.30
10	06-0003	50	26.5	Abdomen	T-LS	3.6
11	06-0006	42	24.2	Abdomen	T-LS	4.8
12	06-0007	46	30.5	Abdomen	T-LS	9.7
13	03-0010	24	24.4	Hip/thigh	T-LS	0.30
14	04-0004	40	ND	Hip/thigh	T-LS	0.82
15	04-0008	27	26.6	Hip/thigh	T-LS	0.16
16	04-0009	36	ND	Hip/thigh	T-LS	0.16
17	05-0005	34	22.2	Hip/thigh	T-LS	0.60
18	05-0006	28	23.2	Hip/thigh	T-LS	0.21
19	05-0008	43	24.6	Hip/thigh	T-LS	7.20
20	06-0005	33	23.2	Hip/thigh	T-LS	0.10
21	06-0010	52	26.2	Hip/thigh	T-LS	2.70
22	06-0012	42	23.7	Hip/thigh	T-LS	0.16

Limiting dilution assay

To assess the frequency of ASCs in the SVF of adipose tissue, SVF cells were seeded in normal culture medium, consisting of DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen, Gibco, Calif., USA) in 96-well plates at 15×10^3 cells/well in the upper row. Two-fold dilution steps of the cells were made in subsequent rows. All cultures were performed in duplicate. Medium was changed twice a week. After 3 weeks, each well was individually scored for the number of cells. A well containing a cluster of at least 10 adhered fibroblast-like cells was considered as being positive. The frequency of ASCs was calculated from the rows of cells for which 25%–75% of the wells were scored as positive.

CFU assays

CFU assays were performed to check the consistency of the limiting dilution assay method and to determine the frequency of CFU capable of differentiating into the osteogenic lineage from the abdomen (n=7) and hip/thigh region (n=6). SVF cells were resuspended in normal culture medium, consisting of DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen, Gibco). Two 6-well plates were prepared in which the SVF was diluted ten-fold across both columns, resulting in a upper column containing 10^4 and a lower column containing 10^3 nucleated SVF cells.

For the CFU-fibroblast (CFU-F) assay, the fixation time was 11–14 days, depending on the amount and growth kinetics of the colonies (merging of colonies was avoided). At the appropriate time point, the medium was removed, and the cells were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 10 min, and subsequently colored in a 0.2% toluidine blue solution in borax buffer for about 1 min. Excess stain was washed off with distilled water, and colonies were counted.

Cells of the duplicate 6-well plate were submitted to a CFU-alkaline phosphatase (CFU-ALP) assay. Cultures were performed in normal medium for 7 days in order to obtain colonies and to remove contaminating cells, after which osteogenic medium was added for 2 weeks. Following this period, cells in the CFU-ALP plate were rinsed with PBS, fixed in 4% formaldehyde, and incubated for 10 min in a 0.2 M TRIS-hydrochloride (pH 10), 0.2 M calcium chloride, 0.1 M magnesium chloride solution, whereafter a solution containing 0.2 M TRIS-hydrochloride (pH 10), 0.2 M calcium chloride, 0.1 M magnesium chloride, and 600 μl nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate was added for 30 min. The percentage of the colonies staining positive for ALP was determined.

Culturing of SVF cells

ASCs from the abdomen and hip/thigh region were cultured up to passage 2 for an adequate and quantitative comparison of stem-cell proliferation and differentiation capacity. Single-cell suspensions of cryopreserved SVF cells were seeded at 5.0×10^6 nucleated cells/cm^2 in normal culture medium. The cultures were maintained in a 5% CO_2 incubator at 37°C in a humidified atmosphere. The medium was changed twice a week. When reaching 80%–90% confluency, cells were detached with 0.5 mM EDTA/0.05% trypsin (Invitrogen) for 5 min at 37°C and replated. Cell viability was assessed by using the trypan blue exclusion assay. A homogeneous population of ASCs was thus obtained from abdomen and hip/thigh region and was subsequently checked by determining growth kinetics and by analyzing the surface-marker expression profile of the ASCs.

Growth kinetics of ASCs

To determine the growth kinetics of cultured ASCs, ten T25 flasks per donor were seeded with 1×10^5 cultured ASCs (passage 2 or 3). At several time points (between days 2 and 12) after seeding, cells from two duplicate flasks were harvested and counted. ASC numbers were plotted against the number of days cultured, and the exponential growing phase of the cells was determined. The population doubling time was calculated by using the formula:

$$Population\ Doubling\ Time = \frac{Days\ in\ exponential\ phase}{(log\ N2 - log\ N1)/log\ 2}$$

where $N1$ was the number of cells at the beginning of the exponential growing phase, and $N2$ was the number of cells at the end of the exponential growing phase.

Flow cytometry

Single-cell suspensions of cultured ASCs from abdomen and hip/thigh region were phenotypically characterized by using fluorescence-activated cell sorting (FACS; FACSCalibur, Becton Dickinson, USA) as previously described (Varma et al. 2007). All monoclonal antibodies (mAbs) were of the immunoglobulin G1 (IgG1) isotype. Cells were stained with fluorescently labeled antibodies (conjugated to fluorescein isothiocyanate, phycoerythrin, or allophycocyanin) against CD31, CD34, CD45, CD54, CD90, CD106, HLA-DR, and HLA-ABC (BD Biosciences, San José, Calif.), CD166 (RDI Research Diagnostics, Flanders, N.J.), CD105 (Caltag Laboratories, Burlingame, Calif.), CD117 (PharMingen, San Diego, Calif.), and CD146 (Chemicon, Temecula, Calif.). Nonspecific fluorescence was determined by incubating the cells with conjugated mAb anti-human IgG1 (DakoCytomation, Glostrup, Denmark).

Chondrogenic and osteogenic differentiation

The chondrogenic and osteogenic differentiation capacities of the cultured ASCs from the abdomen (n=4) and hip/thigh region (n=4) were studied. Chondrogenic differentiation was induced in cultured ASCs as previously described, with some modifications (Oedayrajsingh-Varma et al. 2006). In short, a 50-µl drop of a concentrated ASC cell suspension (8×10^6cells/ml, passage 2) was applied to a glass slide and allowed to attach at 37°C for 1 h. Then, 750 µl chondrogenic medium, consisting of DMEM, plus ITS+ Premix (final concentration in medium when diluted 1:100 was 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenous acid, 1.25 mg/ml bovine serum albumin (BSA), 5.35 µg/ml linoleic acid; BD, USA), 10 ng/ml transforming growth factor- β_1 (TGF-β_1; Biovision, ITK-diagnostics), 1% FCS, 25 µM ascorbate-2-phosphate (Sigma, St. Louis, Mo.), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, was overlaid gently. Cells were maintained in a 5% CO_2/1% oxygen custom-designed hypoxia workstation (T.C.P.S. Rotselaar, Belgium) at 37°C in a humidified atmosphere, as this was shown to enhance chondrogenic differentiation (data not shown). Chondrogenic media were changed every 2–3 days.

For osteogenic differentiation, ASCs (passage 2) were seeded at 5000 cells/cm^2 and cultured in monolayer in osteogenic medium, consisting of normal culture medium supplemented with 10 mM β-glycerol phosphate, 50 µg/ml ascorbate-2-phosphate, and 100 ng/ml bone morphogenetic protein 2 (BMP-2, Peprotech EC, London, UK). Osteogenic medium was changed twice a week.

(Immuno)histochemistry

(Immuno)histochemistry was performed as described previously (Oedayrajsingh-Varma et al. 2006). Cell nodules that formed under chondrogenic culture conditions were stained with Alcian blue (Sigma-Aldrich, Zwijndrecht, The Netherlands) at acidic pH for detection of proteoglycans. For the detection of collagen Type II, staining was performed with mouse monoclonal antibody II-II6B3 (1:50; Developmental Studies Hybridoma Bank, Iowa, USA) against human collagen Type II in PBS containing 1% BSA.

Osteogenesis was visualized after 21 days of culture in osteogenic medium by Von Kossa staining to establish the formation of a calcified matrix, typical for mature osteoblasts. The protocol used was as described previously (Varma et al. 2007) with only one modification of counter-

staining the cytoplasm of the cells using fast green. Calcified extracellular matrix was visualized as black spots.

Spectrophotometric ALP activity

Early differentiation of MSCs into immature osteoblasts is characterized by ALP enzyme activity, with human MSCs expressing ALP as early as 4 days after induction, and maximum levels being observed at around 14 days after induction (Jaiswal et al. 1997). Therefore, cellular ALP activity was measured after culturing the ASCs in osteogenic medium for 14 days. Cells were lysed with distilled water, and the ALP activity and protein content were determined. To determine ALP activity, p-nitrophenyl phosphate (Merck, Darmstadt, Germany) at pH 10.3 was used as the substrate, as described by Lowry (1955). ALP activity was expressed as micromole per microgram of protein in the cell layer. The amount of protein was determined by using a BCA Protein Assay reagent Kit (Pierce, Rockford, Ill., USA), and the absorbance was read at 540 nm with a microplate reader (Biorad Laboratories, Hercules, Calif., USA).

Real-time polymerase chain reaction

RNA isolation and reverse transcription were performed as previously described (Oedayrajsingh-Varma et al. 2006). Real-time polymerase chain reactions (PCR) were performed by using the SYBRGreen reaction kit according to the manufacturer's instructions (Roche Diagnostics) in a LightCycler 480 (Roche Diagnostics). cDNA (approximately 5 ng) was used in a volume of 20 µl PCR mix (LightCycler DNA Master Fast Start^{plus} Kit, Roche Diagnostics) containing a final concentration of 0.5 pmol primers. Relative housekeeping gene expression for 18 S-rRNA (18 S) and relative target gene expression for aggrecan (AGG), collagen Type II (COL2B), and collagen Type X (COL10α1) regarding chondrogenic differentiation, and for collagen Type I (COL1α), osteopontin (OPN), and runt-related transcription factor 2 (RUNX-2) regarding osteogenic differentiation were determined.

Primers (Invitrogen) used for real-time PCR are listed in Table 2. They were designed by using Clone Manager Suite software program version 6 (Scientific & Educational Software, Cary, N.C., USA). The amplified PCR fragment extended over at least one exon border, based on homology in conserved domains between human, mouse, rat, dog, and cow, except for the 18 S gene (encoded by one exon only). Amplified Col2B PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. For real-time PCR, the values of relative target gene expression were normalized to relative 18 S housekeeping gene expression.

Real-time PCR data analysis

With the Light Cycler software (version 4), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene by the Fit Points method. PCR efficiency was calculated by Light Cycler software, and the data were used only if the calculated PCR efficiency was between 1.85–2.0.

Statistics

Kolmogorov-Smirnov tests were used to determine the normalcy of measurements and, if appropriate, their logarithmics. For the evaluation of yield and growth kinetics, means between two groups in one variable were compared by using the independent sample two-tailed *t*-

Table 2 PCR primer sets used for reverse transcription/PCR (*18 S* 18 S subunit, *AGG* aggrecan, *COL1αI* collagen Type I, *COL2B* collagen Type II, *COL10a1* collagen Type X, *OPN* osteopontin, *RUNX-2* runt-related transcription factor 2)

Gene	Primer sets	Accession number, product length (bp)
18 S	Forward: 5′ GTAACCCGTTGAACCCCATT– 3′ Reverse: 5′ CCATCCAATCGGTAGTAGCG 3′	Human, NM_10098, 151 bp
AGG	Forward: 5′CAACTACCCGGCCATCC 3′ Reverse: 5′GATGGCTCTGTAATGGAACAC 3′	Human, NM_001135, 160 bp
COL1αI	Forward: 5′ AAGCCGAATTCCTGGTCT 3′ Reverse: 5′ TCCAACGAGATCGAGATCC 3′	Human, NM_000088, 195 bp
COL2B	Forward: 5′ AGGGCCAGGATGTCCGGCA 3′ Reverse: 5′ GGGTCCCAGGTTCTCCATCT 3′	Human, NM_033150, 195 bp
COL10a1	Forward: 5′ CACTACCCAACACCAAGACA 3′ Reverse: 5′ CTGGTTTCCCTACAGCTGAT 3′	Human, NM_000493, 225 bp
OPN	Forward: 5′ TTCCAAGTAAGTCCAACGAAAG 3′ Reverse: 5′ GTGACCAGTTCATGAGATTCAT 3′	Human, AF_052124, 181 bp
RUNX-2	Forward: 5′ ATGCTTCATTCGCCTCAC 3′ Reverse: 5′ ACTGCTTGCAGCCTTAAAT 3′	Human, NM_001024630, 156 bp

test. Partial correlation was expressed as the Pearson correlation coefficient, r. For evaluation of gene expression, a repeated measures analysis of variance was used to determine significant differences when increasing time-points in one donor within one variable were compared. If levels of gene expression were below the detection limit (0.05), values were set at 10^{-2} (or log level at −2). All statistical tests used a significance level of α=0.05.

Results

Effects of tissue-harvesting site on frequency of ASCs

In a previous study, we demonstrated that the yield of nucleated cells in the SVF of adipose tissue from different tissue-harvesting sites was similar (Oedayrajsingh-Varma et al. 2006). To investigate whether the tissue-harvesting site affected the frequency of ASCs in the SVF in the present study, limiting dilution and CFU-F assays were performed. The outcomes of both types of assays were similar (Fig. 1a, b). When combined, the SVF of adipose tissue harvested from the abdomen contained 5.1±1.1% ASCs (mean± SEM), whereas the percentage of ASCs in the SVF of adipose tissue harvested from the hip/thigh region was much lower (1.2±0.7%; Fig. 1c). This difference in ASC frequency between adipose tissue from the abdomen and hip/thigh region was significant (P=0.0009).

Effect of tissue-harvesting site on frequency of CFUs having osteogenic differentiation potential

Parallel to the CFU-F assay (Fig. 1b), CFU-ALP assays were also performed to determine the percentage of the CFUs capable of osteogenic differentiation. For adipose tissue from the abdomen, 54.9±12.1% (mean±SEM) of the CFUs stained positive for ALP, whereas the CFUs from adipose tissue of the hip/thigh region displayed an ALP positivity of 72.8±8.1% (Fig. 2). Apparently, no differences in osteogenic potential existed between ASCs from the two tissue-harvesting sites (P=0.43).

Phenotypic characterization and growth kinetics of cultured ASCs

Cultured ASCs (passages 2 to 4) from abdomen and hip/thigh regions were phenotypically characterized. ASCs from both tissue-harvesting sites were demonstrated to be homogeneous populations staining positive for stem-cell-associated markers CD34, CD54, CD90, CD105, CD166, and HLA-ABC, and negative for hematopoietic/leukocytic/endothelial markers such as CD31, CD45, CD106, CD146, and HLA-DR (Table 3).

To determine growth kinetics, the population doubling time of ASCs from passages 2 to 3 was determined. When ASCs numbers were monitored over time, a cell growing curve was obtained showing an exponential growing phase, after which the cells reached confluency (Fig. 3a,b). The mean population doubling time of the ASCs in the exponential growing phase was about 2 days when the adipose tissue was harvested from the abdomen and hip/thigh regions (abdomen: 2.1±0.8; hip/thigh: 2.3±0.3; mean±SEM; Fig. 3c).

Effect of tissue-harvesting site on osteogenic differentiation potential of ASCs

Differentiation of cultured ASCs into the osteogenic lineage was induced by culturing the cells in monolayer in

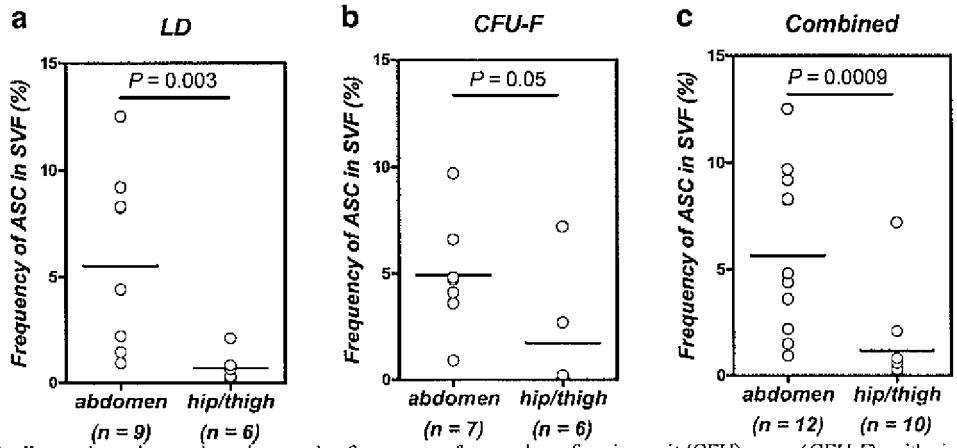

Fig. 1 Effect of adipose-tissue-harvesting site on the frequency of adipose-derived stem cells (*ASCs*) in the stromal vascular fraction (*SVF*). After isolation of the SVF from adipose tissue of both tissue-harvesting sites, the frequency of ASCs in the SVF isolates was determined by using: (**a**) a limiting dilution assay (*LD*) and (**b**) a colony-forming unit (CFU) assay (*CFU-F*), with similar results. When combined (**c**), a significant difference was detected in ASC frequency between adipose tissue harvested from the abdomen and adipose tissue harvested from the hip/thigh region (*P*-values: **a** limiting dilution: P=0.003, **b** colony-forming unit: P=0.05, **c** combined: P=0.0009)

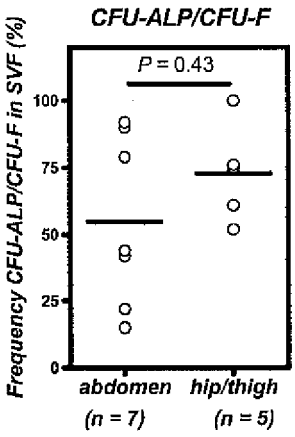

Fig. 2 Effect of adipose-tissue-harvesting site on the osteogenic diffentiation capacity of CFU from the abdomen or hip/thigh regions. No significant difference is apparent in the CFU-alkaline phosphatase (*CFU-ALP*) frequency from the abdomen and hip/thigh region when corrected for CFU-fibroblast (*CFU-F*).

osteogenic medium containing BMP-2. Specific RNA expression of osteogenically induced ASCs from all donors tested increased over time, RUNX-2 being up-regulated 18-fold (P=0.002) and COL1α being up-regulated seven-fold (P=0.024) after 7 days (Fig. 4a,b). OPN gene expression was increased after 7 days; however, this increase was not significant (Fig. 4c). No significant differences were detected in osteogenic gene expression between ASCs derived from the abdomen and ASCs derived from the hip/thigh region, at all three time points tested.

ALP activity in the ASCs was measured after 14 days of osteogenic stimulation. ASCs cultured in control medium served as negative controls. ALP activity in the osteogenically stimulated cells was significantly increased (P=0.047) compared with that in control cells (Fig. 4d). No statistically significant difference was apparent in ALP activity between ASCs derived from abdominal fat and ASCs from hip/thigh fat. Calcification of the osteogenic matrix was confirmed by using Von Kossa staining; black spots could be observed after 3 weeks of osteogenic stimulation of ASCs from both origins (Fig. 4e: abdomen, Fig. 4f: hip/thigh). No calcification was seen in ASC cultures expanded in control medium (Fig. 4g).

Table 3 Surface-marker expression of human cultured ASC at passages 3–4. Results are expressed as mean fluorescence (MF), with isotype control <4.25 (− MF<8.5; + 8.5<MF<100; ++ 100<MF<1000; +++ MF>1000).

Cell-surface marker	Cultured ASC (n=4)
CD29	++
CD31	−
CD34	+
CD45	−
CD54	++
CD90	+++
CD105	++
CD106	−
CD146	−
CD166	+
HLA-ABC	++
HLA-DR	−

Effect of tissue-harvesting site on chondrogenic differentiation potential of ASCs

The chondrogenic differentiation potential of ASCs was analyzed after culturing the cells in a micromass in chondrogenic medium containing TGF-β. Within 24 h of culture, most of the cells formed nodules (n=7).

PCR-amplified COL2B mRNA expression was detectable but not quantifiable after 7 days in ASCs from both the abdomen and hip/thigh region in most but not all donors (n=5). As shown in Fig. 5a, cells from both tissue-harvesting sites displayed COL2B mRNA. Under non-chondrogenic conditions, no COL2B could be detected. AGG and COL10α1 mRNA expression in all donors tested increased over time, AGG being up-regulated 2.4-fold (P=0.041) at day 7 when compared with day 4, and COL10α1 being up-regulated four-fold (P=0.024) after 7 days (Fig. 5b,c). No significant differences were detected in chondrogenic gene expression between ASCs derived from the abdomen and

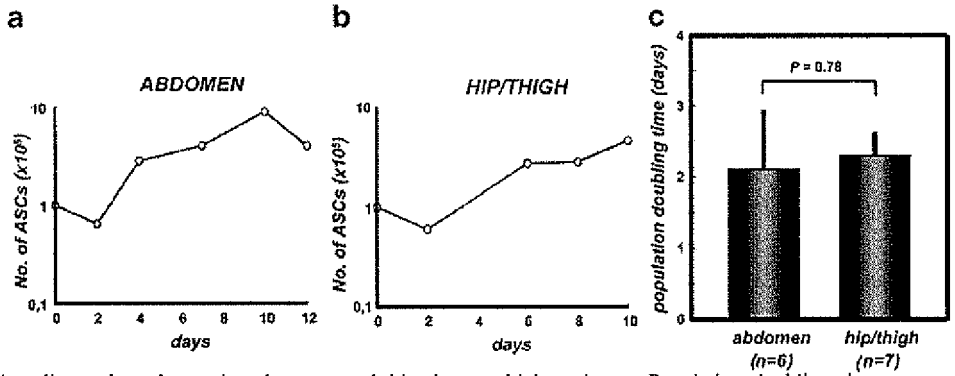

Fig. 3 Effect of the adipose-tissue-harvesting site on growth kinetics of ASCs in vitro. **a** Growth kinetics of ASCs of a representative donor when adipose tissue was harvested from the abdomen. **b** Growth kinetics of ASCs when adipose tissue was harvested from the hip/thigh region. **c** Population doubling time was calculated from the exponential growing phase of the cells. There was no significant difference in population doubling time of ASCs from the abdomen and hip/thigh region (P=0.78, independent Student t-test).

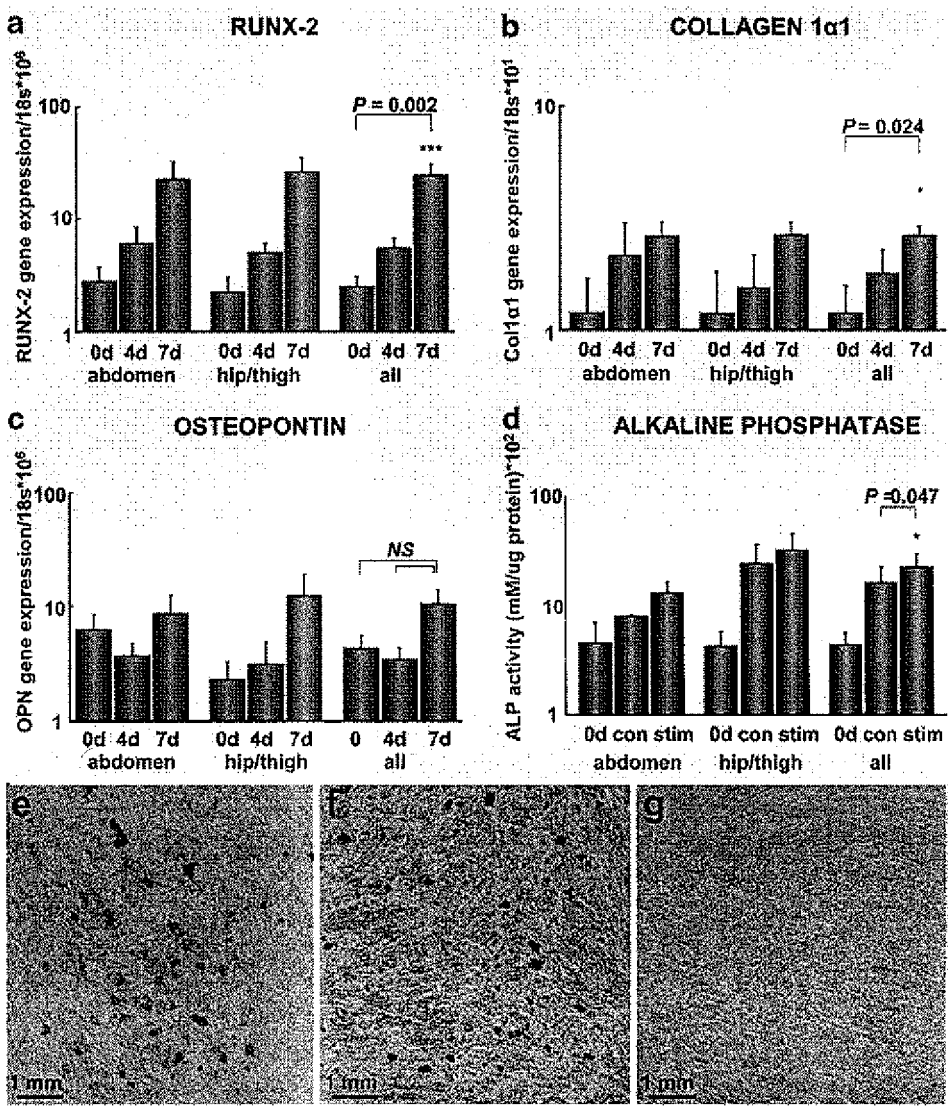

Fig. 4 Effect of adipose-tissue-harvesting site on the osteogenic differentiation of cultured ASCs in vitro. **a–c** *RUNX-2* (runt-related transcription factor 2; $P=0.002$), *COL1α1* (collagen type Ia; $P=0.024$), and *OPN* (osteopontin; $P=0.38$) gene expression was measured after 0, 4, and 7 days (*d*) of osteogenic induction, by using quantitative real-time polymerase chain reaction (qRT-PCR). No significant differences were detected in osteogenic gene expression between ASCs derived from the abdomen and ASCs derived from the hip/thigh region, at all three time points tested. **d** ALP activity was significantly increased after 14 days in the osteogenically stimulated cells (*stim*) compared with that in unstimulated cells (*con*; $P=0.047$). No statistically significant difference was apparent in ALP activity between ASCs derived from abdominal fat and ASCs from hip/thigh fat. **e–g** Von Kossa staining of ASCs from abdomen (**e**) and hip/thigh region (**f**) after 21 days of culture in osteogenic medium and in control medium (**g**), showing mineralized matrix visible as *black spots*.

ASCs derived from the hip/thigh region, at all three time points tested.

Alcian blue staining demonstrated proteoglycan deposition in the ASC nodules from both the hip/thigh region and abdomen (Fig. 5d). At higher magnification (Fig. 5e), the ASC nodules resembled cartilage-like tissue, composed of round cells, surrounded by lacunae and lying in a proteoglycan-rich extracellular matrix that appeared positive for collagen Type II by immunostaining (Fig. 5f).

Discussion

In this study, we have investigated whether the yield and functional characteristics of ASCs are affected by the adipose tissue-harvesting site, i.e., abdomen and hip/thigh regions. We have found a difference in the frequency of ASCs between adipose tissue harvested from the abdomen and the hip/thigh regions. SVF isolates derived from abdominal fat contain significantly higher frequencies of ASCs. When cultured, the growth kinetics and surface-marker expression of ASCs from both tissue-harvesting sites are similar. We have detected no differences in osteogenic or chondrogenic differentiation potential between these cultured ASCs from the two tissue-harvesting sites.

Adipose tissue is a highly heterogeneous tissue, not only among individuals, but also when comparing different fat depots within one individual. Donor-dependent differences have been demonstrated to exist in (stem) cell yield, proliferation, and differentiation capacity, probably caused by differences in age (Hauner and Entenmann 1991; van Harmelen et al. 2003), BMI (Aust et al. 2004; Hauner et al. 1988; Jaiswal et al. 1997; van de Venter et al. 1994), and diseases such as osteoarthritis and diabetes (Barry 2003; Murphy et al. 2002; Ramsay et al. 1995). Donors used in this study for harvesting adipose tissue were healthy female

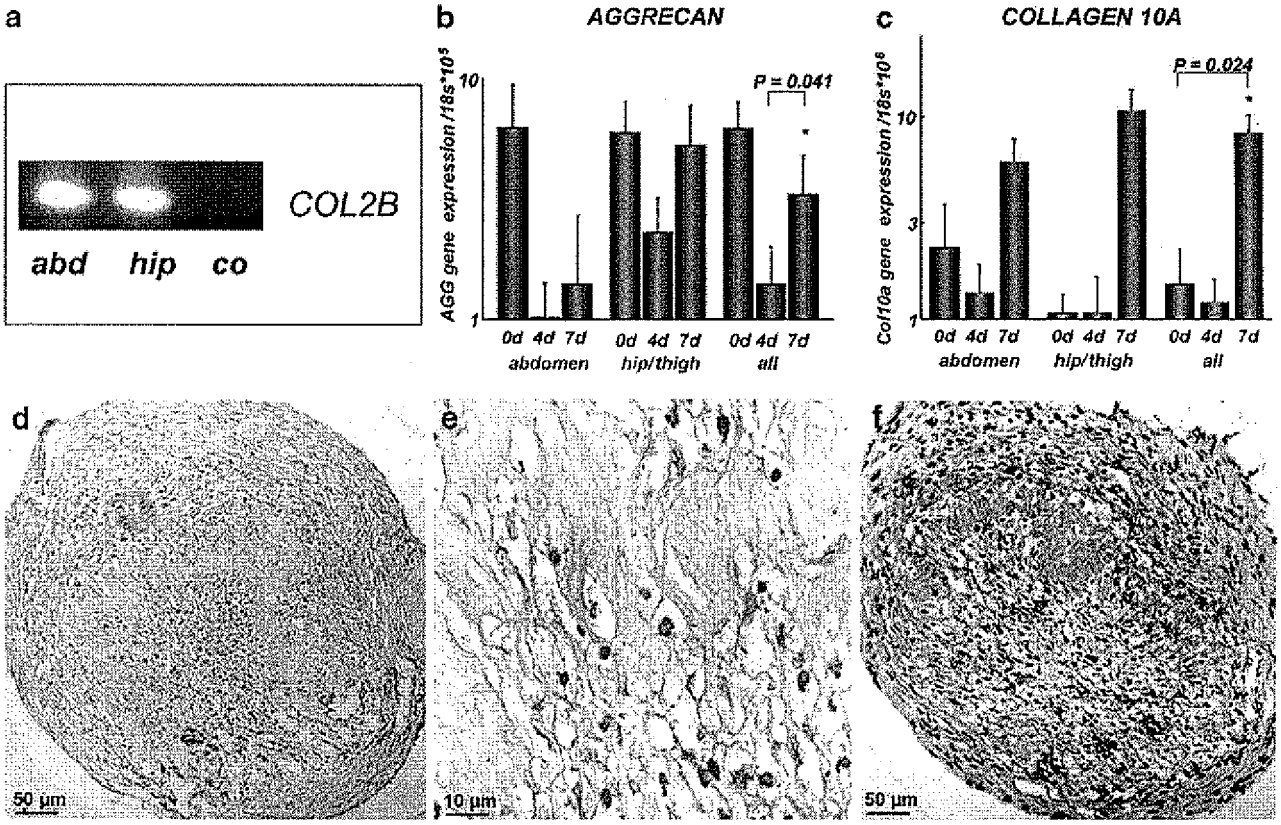

Fig. 5 Effect of the tissue-harvesting site on the chondrogenic differentiation of cultured ASCs in vitro. **a** Both abdomen (*lane 1*) and hip/thigh region (*lane 2*) display COL2B mRNA. Under nonchondrogenic conditions, no COL2B could be detected (*lane 3*). **b**, **c** Aggrecan (*AGG*; $P=0.041$) and collagen 10A (*Col10a*; $P=0.024$) gene expression, respectively, was up-regulated after 7 days (*d*), as measured by qRT-PCR. No significant differences were detected in chondrogenic gene expression between ASCs derived from the abdomen and ASCs derived from the hip/thigh region, at all three time points tested. **d**, **f** Cartilaginous matrix expression was visualized in both tissue-harvesting sites by staining proteoglycans (Alcian blue) and COL2 (Col2-II6B3 antibody), respectively. **e** At higher magnification, the ASC nodules resembled cartilage-like tissue, composed of spherical cells surrounded by lacunae and lying in a proteoglycan-rich extracellular matrix.

donors up to 62 years old. Some patients who will benefit from tissue engineering of cartilage will be older or might suffer from disease, and males will also be affected. Therefore, future research should include these donor types to determine whether yields and functional characteristics are influenced by these variables.

In our donor population, no significant correlation has been detected between the frequency of ASCs and the age of the donor ($P=0.32$, $r=0.27$) or between the frequency of ASCs and BMI ($P=0.42$, $r=-0.22$; data not shown). These data are in agreement with other studies that have demonstrated no correlation between BMI or age and numbers of ASCs per gram of adipose tissue (Hauner and Entenmann 1991; van Harmelen et al. 2003). Most importantly, this means that these variables cannot be responsible for the difference that we have found between the frequency of ASCs and the tissue-harvesting site.

In addition to donor-dependent heterogeneity, intra-individual differences between fat depots have been demonstrated, e.g., with regard to the metabolic response of adipose tissue to various hormonal and neurological stimuli (Guilak et al. 2004; Lacasa et al. 1997; Masuzaki et al. 1995; Monjo et al. 2003; Rodriguez-Cuenca et al. 2005) and the cellular composition of the adipose tissue (Peptan et al. 2006; Prunet-Marcassus et al. 2005). In this study, we have focused on the intra-individual differences in the yield and function of ASCs from two fat depots: the abdomen and the hip/thigh region. We have demonstrated that adipose tissue derived from the abdomen contains significant higher frequencies of stem cells compared with adipose from the hip/thigh region. Whereas SVF isolates from the abdomen in this study contain about 5.1% of ASCs, the frequency of ASCs in SVF from the hip/thigh region is only 1.2%. Moreover, this 1.2% is the average of a population in which some donors hardly possess any adipose-derived stem cells (see spreading ASC frequency hip/thigh in Fig. 1). Despite this more than four-fold difference, the frequency of ASCs in adipose tissue from the hip/thigh region is still much higher compared with the frequency of MSCs in the bone marrow compartment,

which is as low as 0.001%–0.01% (Pittenger et al. 2000), thereby making the hip/thigh region still a much more attractive stem-cell source for tissue engineering therapies.

What are the implications of these stem-cell frequencies for clinical practice? We have shown that $0.5–2.0\times10^8$ SVF cells can be harvested from 100 g adipose tissue, an amount that can easily be obtained from a patient (Aust et al. 2004; De Ugarte et al. 2003; Oedayrajsingh-Varma et al. 2006; Zuk et al. 2001). With an ASC frequency of 5.1%, the SVF isolates contain between $2.6–10.2\times10^6$ stem cells, which is an amount that appears to be sufficient for cell-based therapies as compared with the amount of cells used by others (Erickson et al. 2002; Fan et al. 2006; Williams et al. 2003; Zheng et al. 2006). This implies that time-consuming culturing and expanding steps of the stem cells can be avoided. In comparison, a bone marrow transplant of 100 ml contains approximately 6.0×10^8 nucleated cells (Zuk et al. 2002), of which only 0.001%–0.01% ($0.06–0.006\times10^6$ cells) are stem cells (Pittenger et al. 2000).

To determine the frequency of CFU capable of differentiating into the osteogenic lineage, a CFU-ALP assay has been performed on cells from the abdomen and hip/thigh region. The frequencies of CFU-ALP (±3%) are almost comparable with those of the CFU-F (Figs. 1b, 2). This is higher than the value that Mitchell et al. (2006) have found in their clonogenic assay of freshly isolated stromal cells (±0.5%). However, they have used a different assay method and shorter incubation period, and their frequency increases to the same level (5%) after progressive passaging (Mitchell et al. 2006).

SVF cells have been cultured up to passage 3 to obtain a homogeneous population of ASCs as starting material for differentiation studies toward the chondrogenic lineage. When comparing the growth kinetics of these cultured ASCs from the abdomen and hip/thigh region, the cell doubling time appears to be similar, being approximately 2 days. This is in accordance with the findings of others who have compared the replication rate of adipocyte precursor cells from various tissue-harvesting sites (Hauner et al. 1988; Pettersson et al. 1985; Roncari et al. 1981; Zuk et al. 2001). On the other hand, when comparing SVF cells from omental and subcutaneous fat, van Harmelen et al. (2004) have found a difference in cell proliferation rate; however, this may be explained by differences in methodological approach, since they use stromal cells instead of cultured ASCs. As we have shown that different fat depots contain different numbers of stem cells, these differences in proliferation rate may be caused by differences in initial stem-cell numbers when using SVF cells. This is reflected in the finding that, in our study, SVF cells derived from abdominal fat reach 80%–90% confluency within 5 days, whereas SVF cells derived from adipose tissue of the hip/thigh region take more than 9 days to reach 80%–90% confluency when seeded in the same density (data not shown).

In addition to the determination of growth kinetics, we have phenotypically characterized the cultured ASCs. The surface-marker expression profile is in accordance with those found by others (Mitchell et al. 2006; Schaffler and Buchler 2007; Varma et al. 2007), making both tissue sites fully comparable with each other (Tables 1, 3).

The homogeneous ASC population has been induced to the osteogenic and chondrogenic lineages. Having determined the osteogenic differentiation capacity of ASCs, we have shown significant up-regulation of osteogenic gene expression, ALP activity, and matrix mineralization. Interestingly, although no significant difference has been detected in ALP activity between ASCs from the abdomen and hip/thigh regions, ASCs from the hip/thigh region tend to show higher values of ALP activity after induction. This might be related to the underlying bone tissue, thereby implying that the ASCs of the hip/thigh region are less multipotent and more committed to the osteogenic lineage.

The chondrogenic differentiation capacity of the ASCs has been demonstrated by the up-regulation of AGG and COL10α1 gene expression and the production of matrix proteins. No difference has been detected in the chondrogenic differentiation potential between ASCs from the abdomen and hip/thigh regions. However, in the PCR studies, we have not succeeded in quantifying COL2α and Col2B mRNA expression for any of the donors tested. Others have obviously faced the same difficulty when trying to measure the up-regulation of COL2B genes in MSCs (Huang et al. 2004; Winter et al. 2003; Zuk et al. 2002). Although intending to measure collagen Type X mRNA expression as a hypertrophic and therefore late marker of chondrogenesis, we have noticed that this mRNA is expressed earlier than collagen Type II mRNA. This is surprising, as we would expect stem cells to have to differentiate into chondrocytes before they can become hypertrophic. However, this unexpected hierarchy of chondrogenic gene expression has also been found by Mwale et al. (2006). Moreover, both Mwale et al. (2006) and we have found AGG to be constitutively expressed in MSCs. Because of this constitutive expression of AGG and the early up-regulation of COL10α1, Mwale et al. (2006) warn against using these molecules as markers for chondrogenesis and chondrocytic hypertrophy. We think that these genes can nevertheless be used as markers for differentiation into the chondrogenic lineage, albeit being exclusively shown as the quantitative up-regulation of gene expression, and always in combination with other chondrogenic markers (an awareness of the possible difference in the function of COL10α1 in chondrogenesis in adult stem cells when compared with embryonic stem cells is also necessary).

Our lack of detection of any significant differences in the osteogenic and chondrogenic differentiation potential when comparing ASCs from the two tissue-harvesting sites seems to be in contrast with studies of Hauner and Entenmann 1991) who have found differences in the adipogenic differentiation potential between SVF cells from abdominal and femoral adipose tissue. However, since Hauner and Entenmann 1991) have used fresh SVF cells instead of culture-passaged ASCs, the variation in differentiation potential might be attributable to differences in numbers of ASCs in the SVF isolates of the two regions, as we have shown in this study. Other factors responsible for the variation in differentiation potential can be ascribed to other specific histological characteristics of the adipose tissue at the anatomical site, such as vascularity and amount of fibrous tissue (Lennon et al. 2000; Peptan et al. 2006; Pittenger et al. 2000), and to differences in the regulation of gene expression (Djian et al. 1983; Peptan et al. 2006; Pittenger et al. 2000).

We therefore conclude that the yield of ASCs is dependent on the tissue-harvesting site. In planning the optimal one-stage procedure for the regeneration of cartilage tissue, factors that can positively influence the outcome of the operation must be taken into account. In view of this, the abdomen seems to be preferable to the hip/thigh region for harvesting ASCs.

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EXHIBIT E

Granulocyte colony-stimulating factor mobilized CFU-F can be found in the peripheral blood but have limited expansion potential

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ABSTRACT

Bone marrow mesenchymal stem cells are multipotent cells found lining the bone marrow cavity supporting the growth and differentiation of hematologic progenitors. There is growing evidence that these cells can, under the right circumstances, enter the peripheral circulation. We show that granulocyte colony-stimulating factor mobilized peripheral blood contains cells which form colonies and have a similar fibroblastic morphology (termed CFU-F) to bone marrow mesenchymal stem cells. These cells were found at a very low incidence (0.0002%). Mobilized peripheral blood CFU-F were successfully differentiated into osteogenic and adipogenic lineages. FACS analysis showed that the cells had a similar profile to bone marrow mesenchymal stem cells. Importantly, mobilized peripheral blood CFU-F had limited expansion potential and became senescent 20-25 days after isolation. Mobilized peripheral blood CFU-F also did not have any telomerase activity and displayed significant telomere shortening. The rarity of CFU-F in mobilized peripheral blood and the subsequent pressure to divide in cell culture probably contribute to early cellular senescence. Their potential for use in transplant or gene therapy is, therefore, limited.

Key words: granulocyte colony-stimulating factor, mobilized peripheral blood, telomere, mesenchymal stem cell, bone marrow.

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Introduction

Mesenchymal stem cells were described more than 40 years ago as a subpopulation of cells in the bone marrow that are plastic adherent in a tissue culture environment.[1] They are not a homogenous stem cell population, but contain several multipotent cell types that can differentiate into adipocyte, osteogenic cells, or cartilaginous precursors.[2] Mesenchymal stem cells also have a unique ability to down regulate the immune system with regard to graft versus host disease (GVHD) affecting T, B and NK cell activity and employ soluble as well as direct cell contact mechanisms.[3]

Granulocyte colony stimulating factor (G-CSF) is commonly used to mobilize hematopoietic stem cells from the bone marrow compartment into the peripheral circulation. Whether or not mesenchymal stem cells can migrate or mobilize into the circulation is unclear, but there is growing evidence that this can occur, although at a very low level.[4] Earlier studies in patients receiving G-CSF mobilized stem cell transplants showed that the mesenchymal stem cell population remained of recipient origin post-transplantation, but on a few occasions donor mesenchymal stem cells engrafted.[5-7] Recently, there has been increasing evidence to show that there are multipotent cells in the circulation that have biological traits and morphology that are similar, but not identical, to bone marrow mesenchymal stem cells.[8,9] Two previous studies have successfully used positive selection methods to isolate mesenchymal stem cells from G-CSF mobilized peripheral blood with either CD105 or dense fibrin microbeads.[10,11] Other early attempts to isolate mesenchymal stem cells from peripheral blood using the classical plastic adherence method (with or without G-CSF mobilization) had only limited success.[12-14]

We show the growth characteristics of a plastic adherent cell population which we successfully isolated from mobilized peripheral blood.

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The online version of this article contains a supplemental appendix.

Design and Methods

Isolation of mobilized peripheral blood CFU-F

Briefly, blood from G-CSF treated healthy volunteers was purchased from AllCells, LLC (Emeryville, CA, USA) (*see Online Supplementary Appendix*). The use of all tissue was approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation, placed in Iscove's media with 20% fetal calf serum (FCS) (Hyclone), and plated on plastic culture flasks. Cells were left undisturbed for seven days without media changes and colonies of adherent cells were then noted on the flask. Culture flasks were washed with PBS and the adherent cells passaged by traditional means using trypsin digestion. Cell numbers and population doublings were measured throughout the culture period.

To isolate bone marrow derived mesenchymal stem cells, 10 mL of bone marrow aspirate was obtained from healthy volunteers. Mononuclear cells were prepared as above and plated like those of the mobilized peripheral blood CFU-F.

Differentiation cultures

Approximately two weeks after isolation, mobilized peripheral blood CFU-F were in sufficient number to differentiate and 50,000 cells per well were placed in a 6-well plate with the appropriate media for osteogenic or adipogenic differentiation (*see Online Supplementary Appendix*). The culture period was 21 days, after which cells were washed with PBS, fixed for 10 mins. in 10% buffered formalin at room temperature and stained with Alizarin Red (for osteogenic cells) or Oil Red-O (for adipogenic cells). Undifferentiated cells were washed with PBS and stained with crystal violet.

Telomere analysis

DNA was extracted from 2 million cells by standard methods and 1 microgram digested with the restriction enzymes HinfIII and RsaI (Invitrogen, Carlsbad, CA, USA). Fragments were separated on an 0.5% agarose gel and transferred to positively charged nylon membrane by vacuum. Standard Southern analysis was performed using a digoxigenin (Dig) 5' and 3' end-labeled telomere probe (TTAGGG). Detection was by a horseradish peroxidase labeled anti-Dig antibody (Roche) and chemiluminescence using CDP-STAR® (Roche). Average telomere lengths were determined by scanning the resulting Southern blot. The Telometric software program was used for the analysis.[15]

Results and Discussion

Isolation of mobilized peripheral blood CFU-F

The study shows that colony forming cells can be isolated from G-CSF mobilized peripheral blood by standard plastic adherence. The morphological appearance was identical to that of bone marrow derived mesenchymal stem cells (Figure 1A). On the basis of their appearance and phenotypic analysis these cells were termed mobilized peripheral blood colony forming unit-fibroblasts (mobilized peripheral blood CFU-F). The success of this method was limited because cultures could only be established in 50% of six attempts. The growth curves of three cultures that grew to three weeks are shown in Figure 2. An additional culture appeared as CFU-F, but did not proliferate.

Mobilized peripheral blood CFU-F were shown to have both osteogenic and adipogenic potential (Figure 1B). Attempts at chondrogenic differentiation were only minimally successful indicating that these cells are more limited in ability than typical bone marrow mesenchymal stem cells (*Online Supplementary Figure 1*).

Although there is no single cell surface antigen specific for mesenchymal stem cells, a panel of commonly positive CD markers on bone marrow mesenchymal stem cells was used to evaluate mobilized periph-

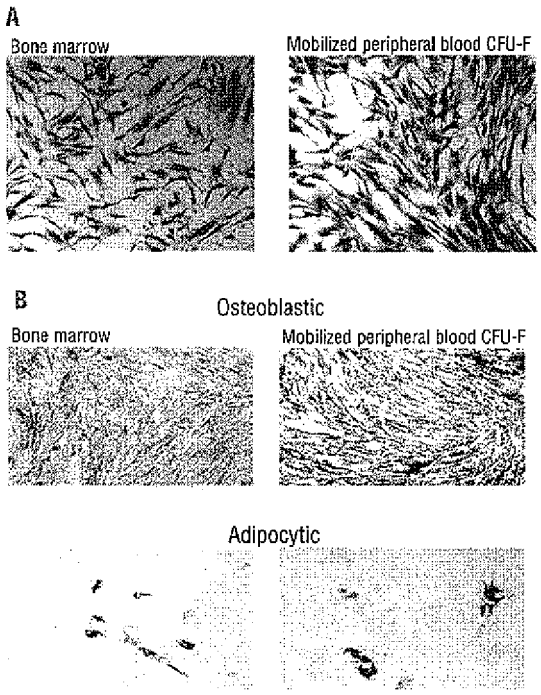
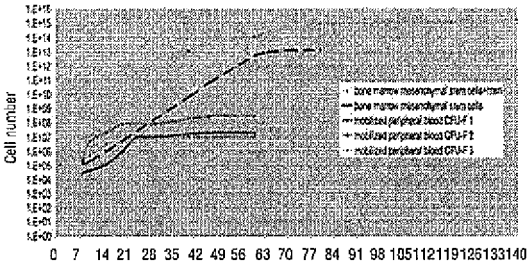


Figure 1. Morphology and differentiation of adherent cells isolated from mobilized peripheral blood. A. Cells were isolated by plastic adherence as described in the Methods section. Adherent cells from bone marrow mononuclear cells were also isolated in an identical fashion and used for comparison. The isolated cells have a triangular and fibroblastoid appearance typical of mesenchymal stem cells. B. Cells at population doubling 5 were placed in a 6-well plate and differentiated for 21 days along osteogenic or adipogenic lines in the appropriate media. At the end of the culture period, cells were washed, fixed, and stained with alizarin red for calcium indicative of osteoblasts or Oil-Red-O, a lipid stain characteristic of adipogenic cells.



	CFU-F	% of starting PBMC	Day 8 cell count
Mobilized peripheral blood CFU-F1	300/10⁶	0.00003	31,500
Mobilized peripheral blood CFU-F2	200/10⁶	0.00002	200,000
Mobilized peripheral blood CFU-F3	1900/10⁶	0.00019	245,000
Bone marrow mesenchymal stem cells	781/10⁶	0.008	130,000

Figure 2. Mobilized peripheral blood CFU-F have limited expansion potential in culture. Cells were isolated from bone marrow or from G-CSF mobilized peripheral blood from healthy donors. Culture initiation was performed as described in the *Design and Methods* section. The graph represents the cumulative number of cells during the culture period. Senescence occurred when the cells undertook no further divisions (curve flattens). A control cell line, (+htert), was taken from a separate experiment with bone marrow mesenchymal stem cells at population doubling 5 via infection with the human telomerase gene *htert* encoded via a retrovirus containing a bicistronic eGFP. Green cells were FACS sorted for eGFP expression and 100,000 cells were used to start a new culture on Day 1. The number of colonies with 30 or more cells counted at day 8 in culture is given in the table below as well as the percentage of the starting cell number.

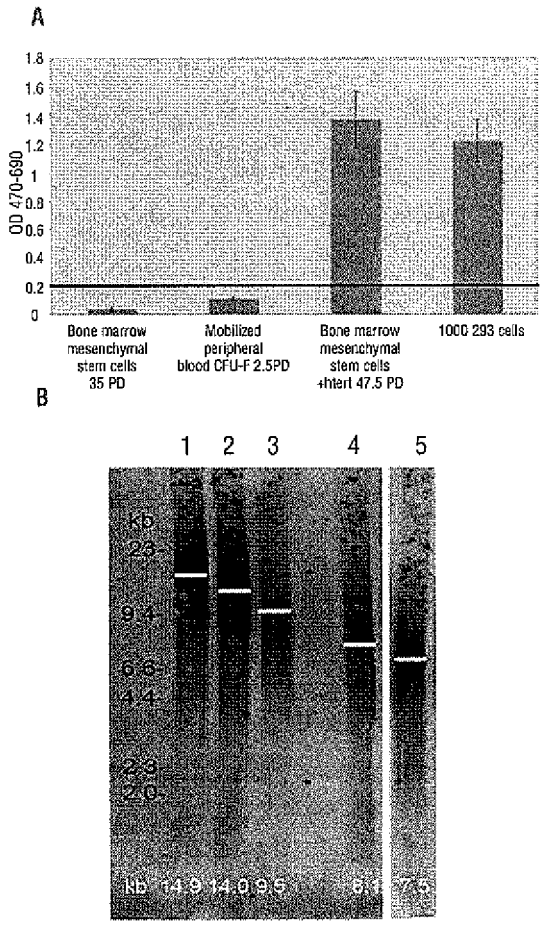


Figure 3. Mobilized peripheral blood CFU-F lack telomerase activity and have shortened telomeres at early onset in culture. A. Protein was prepared from 250,000 cells and normalized by Bradford assay (Pierce). Telomerase activity was evaluated by the TRAPEZE® ELISA kit from Chemicon (Temecula, CA, USA). Units are expressed as OD 470–OD 690 nm absorbance. Background was determined from heat-inactivated extracts and subtracted from total activity. Samples with OD 470-690 >0.2 are considered to possess telomerase activity (red line) (n=4). Extracts from the 293 cell line and bone marrow mesenchymal stem cells+htert (bone marrow mesenchymal stem cells harboring the human telomerase gene) cell line were used as positive controls. B. Cells were harvested at the population doubling (PD) as noted and DNA prepared by standard methods. A Southern blot was performed using a labeled telomere probe: (TTAGGG)3. The white bar indicates the mean telomere length determined by unidirectional signal strength averaging using Telometric software (actual kb values are listed below each lane). Lane 1 is bone marrow mesenchymal stem cells+htert, lanes 2 and 3 are bone marrow mesenchymal stem cells PD 4 and 35 respectively, lane 4 is mobilized peripheral blood CFU-F1 at PD 5, and lane 5 is mobilized peripheral blood CFU-F2 at PD 5.

eral blood CFU-F. The cells were consistently positive for CD13, CD29, CD105 and CD166. Cells were negative for CD14, CD34, CD45 and CD133. This pattern of expression was maintained throughout culture. An identical pattern of expression was seen in our bone marrow derived mesenchymal stem cells and is consistent with the published literature on mesenchymal stem cells[16] (*Online Supplementary Figure 2*).

Growth characteristics of mobilized peripheral blood CFU-F

Enumeration and calculation of frequency show the cells to be at least 50 times less frequent than bone marrow mesenchymal stem cells existing at 0.00003%-0.0002% (Figure 2). The mobilized peripheral blood CFU-F expanded after some initial lag time in culture. After reaching approximately 6,500 cells/cm², mobilized peripheral blood CFU-F entered a more robust state of growth dividing roughly every 48 hrs. for about three weeks until they reached a point of senescence. Bone marrow derived mesenchymal stem cells were also isolated and grown in comparison to the mobilized peripheral blood CFU-F. Bone marrow mesenchymal stem cells continued in culture for a longer duration until reaching senescence at about 35-50 population doublings. There are several other reports of isolating mesenchymal stem cells from human peripheral blood by a variety of methods.[10,11,17] These reports have shown an early exponential expansion of the peripheral blood mesenchymal stem cells, but few comment on longetivity. Work by Zvaifler *et al.* has shown that cells with mesenchymal stem cell morphology and phenotype can be found in the peripheral blood. [18] It was estimated that several thousand mesenchymal stem cells were found in 0.5 liter of peripheral blood which is certainly less than <0.1% of PBMCs. Growth curve data showed evi-

dence of early exponential growth in culture, but was only reported to 17 days and achieved 7 population doublings. It is worth noting that in the work of Kassis *et al.*, mesenchymal stem cells were isolated from G-CSF mobilized peripheral blood, but could not grow past 18 days in culture.[10] Our growth curve data agree with both these observations. Lazarus *et al.* attempted to isolate mesenchymal stem cells from G-CSF mobilized peripheral blood but was unsuccessful. This was possibly due to the small volumes used (only 10 mL of blood).[14]

Whether CFU-F truly migrate from the bone marrow is difficult to prove. Several groups have shown that there are cells circulating which have mesenchymal stem cell-like properties and can contribute to organ repair when evaluated under injury or transplant conditions.[19] The native migration of PB CFU-F is unknown, but if they are present in the peripheral circulation they are likely to be very rare indeed. We did not find any CFU-F in non-G-CSF treated peripheral blood. While G-CSF allowed CFU-F identification to be made, it is possible that other cytokines such as interleukin-8 may be more effective for their mobilization.[20]

Mobilized peripheral blood CFU-F lack telomerase and have short telomeres

Typically, bone marrow mesenchymal stem cells lack telomerase and have a defined lifespan of 30-50 population doublings. Neither our bone marrow mesenchymal stem cells or mobilized peripheral blood CFU-F showed any detectable telomerase activity. We also aimed to determine if there was expected telomere erosion *in vitro*. In evaluating telomere length, bone marrow mesenchymal stem cells at an early population doubling have relatively long telomeres at 14 kb, while those at higher population doublings show significant shortening (Figure 3B). Mobilized peripheral blood CFU-F from two samples were evaluated at an early population doubling and have approximately 50% shorter telomeres than their bone marrow counterparts.

It has been reported that bone marrow stroma cells isolated from older individuals have a decreased life span and undergo senescence earlier than those from younger people.[21] In addition, bone marrow mesenchymal stem cells do not express telomerase de novo, but ectopic telomerase expression can be induced to extend the lifespan of these cells while retaining their differentiation potential.[22] When evaluating the telomere length in peripheral blood mononuclear cells, some correlation to donor age has been shown.[23] We do not have that information for our cells (only that donors were over 18 years of age and in good health). Our culture outgrowths underwent significant cell divisions and subsequently lost telomeres for two main reasons. First, the cells were placed into optimal culture conditions, and second and more importantly, they are very rare in number and therefore under tremendous pressure to divide. Further population doublings of mobilized peripheral blood CFU-F could not be analyzed as cell quantities were limited. The significant telomere shorting probably contributes to mobilized peripheral blood CFU-F early cell senescence, although this could also be influenced by donor age. An ideal experiment would involve isolating both marrow derived mesenchymal stem cells and mobilized peripheral blood CFU-F from the same donor, but availability of this type of donation would be unlikely.

Despite limited ability to expand *in vitro*, one may theorize that the presence of mesenchymal stem cells in the peripheral circulation in times of organ damage/injury serves a specific purpose. Cells could be actively recruited to damaged tissues (via appropriate cytokines) and promote healing/repair as well as the recruitment of other cell types. Because mesenchymal stem cells are potent secretors of many cytokines, they may have a significant biological effect even in small cell numbers.[15,24]

While our isolation of CFU-F from mobilized peripheral blood is not necessarily novel, it does underlie the difficulty of isolating mobilized peripheral blood CFU-F using the current standard method of plastic adherence (our success rate was only 50%). This may be quite a significant limitation if these types of cells are ever to be considered for clinical studies. Additionally, their lack of expansion and limited differentiation show they have restricted potential, and although they share surface markers with bone marrow mesenchymal stem cells, they may, in fact, be different cells altogether.

In conclusion, we have shown that G-CSF mobilized PBMCs contain a population of cells with classical phenotypic markers of mesenchymal stem cells and limited differentiation properties. These cells are properly termed mobilized peripheral blood CFU-F. They are very rare in G-CSF mobilized peripheral blood and display limited expansion *in vitro*, most likely due to early cell senescence secondary to significant telomere erosion which will limit their potential for future use in the clinical arena.

Authorship and Disclosures

TL: performed all experiments and wrote manuscript; JT: key experimental design and critical reading; PO: laboratory PI. The authors reported no potential conflicts of interest.

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EXHIBIT F

Cell Tissue Kinet. (1985) **18**, 589–595.

Migration of fibroblastoid stromal cells in murine blood

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Abstract. This paper describes the kinetics of fibroblastic colony forming units (CFU-f) in murine blood after phenylhydrazine-induced haemolytic anaemia and their subsequent migration into haemopoietic organs. Murine blood contained 5·3 ± 0·8 CFU-f per 10^6 nucleated cells. Absence of particle ingestion and factor VIII-related antigen in addition to the enzyme pattern in CFU-f-derived cells confirmed that these cells did not have a macrophage-like or endothelial nature. Phenylhydrazine treatment of mice resulted in a 3-fold increase in blood CFU-f numbers which was accompanied by increases in blood cellularity and granulocyte-macrophage progenitor numbers. When both partners of CBA/N and CBA/T6T6 mice in parabiosis had been treated with phenylhydrazine, spleens and femoral bone marrow of both mice were shown to contain partner-derived CFU-f. These data suggest that circulating CFU-f represent a stromal cell population which can migrate into haemopoietic organs.

The presence of fibroblastoid cells in the blood stream has been demonstrated in guinea pigs (Maximow, 1928), humans (Paul, 1958), mice (Metcalf, 1972), rabbits (Friedenstein, 1976) and dogs (Klein *et al.*, 1983). The possibility that these cells were released from blood vessel walls during blood sampling was excluded by Luria *et al.* (1971). Circulating fibroblastic cells may represent a stromal cell population that migrates between tissues and organs; however, conclusive evidence for this concept has so far not been presented (Friedenstein, 1976).

In the present study we examined the kinetics of fibroblastic colony-forming units (CFU-f) in blood, spleen and bone marrow of mice after induction of haemolytic anaemia with phenylhydrazine. Migratory properties of CFU-f were then studied by treating normal and chromosome-marked mice in parabiosis with phenylhydrazine and assessing the origin of spleen and bone marrow-derived CFU-f afterwards. Thus we provided evidence for migration of CFU-f from one partner to the spleen and bone marrow of the other.

MATERIALS AND METHODS

Male (CBA/Rij × C57BL/Rij)F1 and CBA/N and CBA/T6T6 mice 10–20 weeks old were used. The mice were ether-anaesthetized and bled from the suborbital plexus. Blood was collected in heparinized tubes. Subsequently the mice were killed by cervical dislocation and the spleens and femurs removed using sterile techniques.

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Nucleated blood cells were counted with Bürker counting chambers. Blood cells were separated (Boyum, 1968) in 0·1% methylcellulose and 50% α-medium by unit gravity for 30 min at room temperature to sediment erythrocytes, and the supernatant cells were then collected. The spleens were pressed through a nylon mesh sieve and suspended in 6 ml of buffered salt solution (BSS) containing 5% foetal calf serum (FCS). Femoral marrow cells were obtained by flushing the marrow from femurs, after removal of the epiphyses, in BSS + 5% FCS. Single cell suspensions from spleen and bone marrow were prepared by repeated flushing of the cells through a twenty-three gauge syringe needle. The cells collected from blood, spleen and bone marrow were centrifuged and resuspended in α-medium containing 5% FCS. Nucleated cell counts were performed with a coulter particle counter, prior to dilution to the required cell concentration in α-medium + 5% FCS.

For CFU-f quantitation the cells were cultured in α-medium containing 0·8% methylcellulose and 20% FCS. One ml aliquots of culture medium containing $2–7 \times 10^6$ spleen cells, $2–7 \times 10^5$ bone marrow nucleated cells, or varying numbers of blood nucleated cells were plated in 35 mm Costar culture dishes and incubated at 37°C in an atmosphere consisting of 10% CO_2 in air. All determinations were performed in triplicate. On day 10 of culture the dishes were rinsed with phosphate buffered saline, fixed in methanol and stained with 10% Giemsa. Fibroblastoid colonies containing at least fifty fibroblastoid cells were counted with an inverted microscope.

Particle ingestion and enzyme stains were performed as described (Piersma *et al.*, 1985). Factor VIII-related antigen was determined with horse-radish-peroxidase conjugated to rabbit-anti-human factor VIII-related antigen (DAKO, Denmark), which cross-reacted with endothelium in frozen sections of various F_1 murine organs at a 1:10 dilution. Binding of the antibody conjugate was detected with diaminobenzidine.

For CFU-gm quantitation the cells were cultured in a 10-fold lower concentration than in the CFU-f assay in α-medium supplemented with 10% FCS, 1% BSA and 10% murine Con-A-spleen-conditioned medium in duplicate for 7 days in an atmosphere consisting of 10% CO_2 in air at 37°C, prior to counting colonies containing at least fifty cells using an inverted microscope.

Haemolytic anaemia was induced by four daily intraperitoneal injections of 1 mg phenylhydrazine-hydrochloride (Merck, Darmstadt, Germany) in 0·5 ml BSS. Mice in parabiosis received two daily injections 1 week after parabiosis. Parabionts were established between CBA/N and CBA/T6T6 chromosome-marked mice according to the method of Benner, van Oudenaren & de Ruiter (1977) and chromosome preparations were performed as previously described (Piersma, Ploemacher & Brockbank, 1983).

RESULTS

In twenty independent experiments, murine blood cells were assayed for CFU-f (Table 1), and were found to contain $5·3 \pm 0·8$ (mean ± SE) CFU-f per 10^6 nucleated cells (range 1·1–13·6). The blood-borne CFU-f colonies consisted of cells with a typical fibroblastoid morphology i.e. large flattened polygonal cells containing a large nucleus with several nucleoli. Furthermore (Table 2) these cells were unable to ingest carbon and latex particles and could not be shown to carry the factor VIII-related antigen, which argues against a macrophage-like or endothelial nature of these CFU-f-derived cells. This point is further strengthened by their enzyme pattern which lacked detectable amounts of non-specific esterase and peroxidase, whereas alkaline phosphatase was weakly positive in a minority of fibroblastic cells within a colonony, and acid phosphatase was found to a limited extent throughout the colonies.

Table 1. CFU-f in murine blood*

Experiment No.	Nucleated cells plated ($\times 10^{-6}$)	No. of CFU-f colonies	CFU-f per 10^6 nucleated cells
1	2·1	6	2·9
2	4·5	51	11·3
3	1·6	19	11·7
4	0·8	11	13·6
5	4·5	11	2·5
6	2·3	6	3·5
7	3·4	4	1·2
8	4·4	13	3·0
9	1·4	3	2·1
10	1·6	8	5·2
11	3·8	17	4·5
12	9·9	67	6·8
13	5·1	45	8·8
14	2·8	18	6·5
15	5·3	29	5·5
16	4·7	21	4·4
17	7·3	25	3·4
18	5·7	6	1·1
19	4·4	16	3·6
20	4·0	17	4·3
mean ± SE			5·3 ± 0·8

* Blood samples taken via the suborbital plexus were pooled from three mice per experiment, and erythrocytes were depleted by methylcellulose agglutination. Remaining nucleated blood cells were washed before plating in culture medium.

Table 2. Characteristics of fibroblastic cells in blood-derived CFU-f colonies

Carbon ingestion	–
Latex ingestion	–
Factor VIII-related antigen	–
Alkaline phosphatase	±
Acid phosphatase	±
Nonspecific esterase	–
Peroxidase	–

In Fig. 1 the nucleated cell numbers of femur, spleen and blood are given for mice that had been treated with four daily injections of phenylhydrazine. Femoral cellularity was not changed, whereas splenic cellularity was increased 3-fold 1 day after treatment and decreased to normal levels between days 5 and 11. Blood cellularity was increased 3- to 4-fold at first and went back to subnormal levels by day 3 and remained as such throughout the study.

After phenylhydrazine treatment, granulocyte/macrophage progenitor cell numbers (Fig. 2) in the femoral bone marrow hardly changed. In the spleen they were elevated by 5-fold, gradually returning to 2-fold levels 11 days after treatment. In the blood these progenitors were increased by 4-fold at the first day after treatment and slowly returned to control values by the eleventh day.

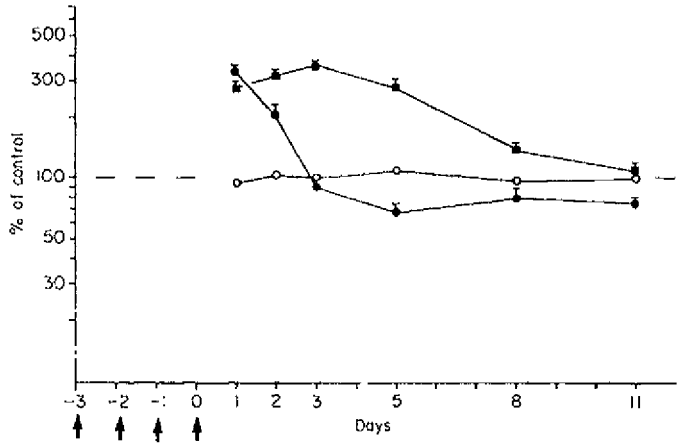

Fig. 1. Nucleated cellularities of femoral bone marrow (○), spleen (■), and 1 ml blood (●) in mice treated with four daily injections (↑) of 1 mg PHZ per 0·5 ml BSS intraperitoneal expressed as percentage of matched untreated controls ± SE of three individual experiments. Each experiment involved three individual mice per point. Control cellularities averaged $5{\cdot}8 \times 10^6$ per ml blood, $2{\cdot}54 \times 10^8$ per spleen, and $2{\cdot}72 \times 10^7$ per femur.

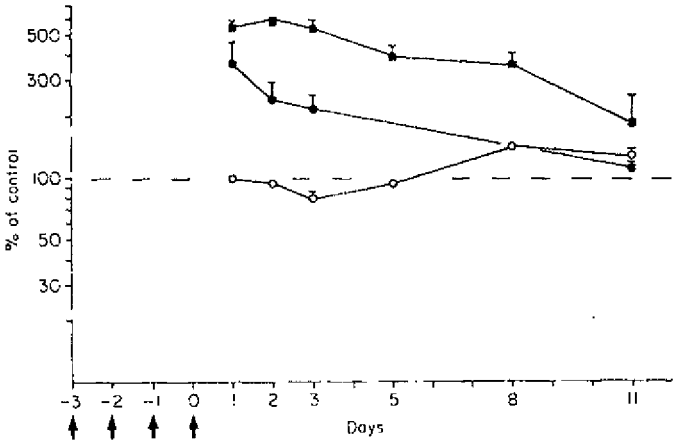

Fig. 2. Granulocyte/macrophage colony-forming units in femoral bone marrow (○), spleen (■) and 1 ml blood (●) in mice treated with four daily injections (↑) of 1 mg PHZ per 0·5 ml BSS intraperitoneal expressed as percentage of matched untreated controls ± SE of three individual experiments. Each experiment involved three individual mice per point. Control CFU gm numbers averaged 57 per ml blood, 9493 per spleen and 37,177 per femur.

Changes in CFU-f numbers following phenylhydrazine treatment are depicted in Fig. 3. In the femur, no significant changes were apparent. In the spleen, a 3-fold increase in CFU-f numbers at day 1 was followed by a decrease to normal levels at day 3. The CFU-f in the blood were variably increased by up to 3-fold throughout the observation period.

In order to establish whether the increase in blood CFU-f numbers reflected a migrating population of stromal progenitor cells in which spleen and/or bone marrow served as target

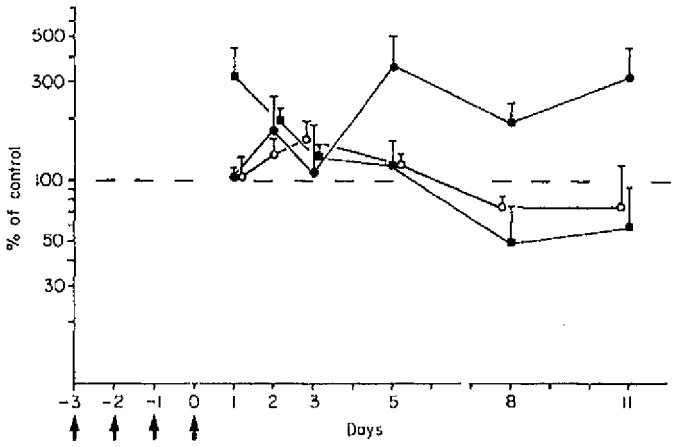

Fig. 3. Fibroblastic colony-forming units in femoral bone marrow (○), spleen (■), and 1 ml blood (●) in mice treated with four daily injections (↑) of 1 mg PHZ per 0·5 ml BSS intraperitoneal expressed as percentage of matched untreated controls ± SE of three individual experiments. Each experiment involved three individual mice per point. Control CFU-f numbers averaged 18 per ml blood, 1262 per spleen, and 1272 per femur.

Table 3. Origin of CFU-f in spleen and bone marrow of parabiosed mice*

Parabiont No.	Partner	Organ	Origin of CFU-f: donor	host	unknown	$\frac{\text{donor}}{\text{donor + host}} \times 100\%$
1	CBA/N	Spleen	0	2	69	0
		Bone marrow	3	10	45	23
	CBA/T6T6	Spleen	4	11	160	27
		Bone marrow	3	13	59	19
2	CBA/N	Spleen	3	13	49	19
		Bone marrow	9	23	56	28
	CBA/T6T6	Spleen	7	17	40	29
		Bone marrow	5	24	37	17
3	CBA/N	Spleen	5	24	72	17
		Bone marrow	13	52	74	20
	CBA/T6T6	Spleen	3	11	37	21
		Bone marrow	9	27	57	25

* One week after parabiosis both partners received two daily injections of 1 mg phenylhydrazine in 0·5 ml BSS and were sacrificed for assay of CFU-f 1 week after this treatment.

organs for CFU-f, we established parabiosis between CBA/N and syngeneic chromosome marked CBA/T6T6 mice. After fusion of the blood streams of the partners, both were treated with phenylhydrazine, and 1 week later the spleens and femoral bone marrow were assayed for donor- and host-derived CFU-f. From Table 3 it can be seen that, with one exception, spleen as well as bone marrow of both partners in three parabionts contained significant amounts of partner-derived CFU-f, indicating that substantial migration from one animal to spleen and bone marrow of the other had occurred.

DISCUSSION

Under steady state conditions murine blood contained $5{\cdot}3 \pm 0{\cdot}8$ CFU-f per 10^6 nucleated blood cells (Table 1). This number is comparable to the data of Luria *et al.* (1971) for guinea pig blood. These authors showed that CFU-f incidence did not change with the number of punctures performed during blood sampling, thus providing evidence that the fibroblastic colonies developed from circulating blood cells. The morphology of the CFU-f colonies and the characteristics listed in Table 2, as well as the absence of the macrophage determinant Mac-1 (Piersma *et al.*, 1985), stress that these cells are not macrophage-like or endothelial in nature.

After induction of haemolytic anaemia by phenylhydrazine treatment blood CFU-f numbers were increased by up to 3-fold, accompanied by an increased cellularity and increased granulocyte-macrophage progenitor numbers (Figs. 1–3). In the spleen similar effects were seen, whereas in the bone marrow these values had hardly changed. These observations confirm and extend studies by Rencricca *et al.* (1970), Hodgson, Bradley & Telfer (1972), Hara & Ogawa (1976) and Ploemacher, van Soest & Vos (1977), who reported on haemopoietic stem and progenitor cell kinetics following phenylhydrazine-induced haemolytic anaemia. Also after endotoxin treatment in mice, splenic CFU-f numbers were greatly increased (Brockbank, Ploemacher & van Peer, 1983). Friedenstein *et al.* (1974) reported an increase of CFU-f numbers in the bone marrow of mice after bleeding and in the lymph nodes of mice after immunization. In partially-irradiated mice the irradiated part of the bone marrow was repopulated by fibroblastoid stromal cells much faster than the bone marrow of total body irradiated mice (Werts *et al.*, 1980). Furthermore, upon intravenous bone marrow transplantation in man (Keating *et al.*, 1982) and mice (Piersma *et al.*, 1983) stromal cells were shown to lodge in the recipient's bone marrow. The need for additional stromal support for haemopoiesis may require influx of stromal cells from other sites via the blood stream. The increase in blood CFU-f numbers after phenylhydrazine treatment supports this concept.

The study with phenylhydrazine-treated parabionts presented here provides conclusive evidence for the influx of blood-borne CFU-f into spleen as well as bone marrow (Table 3). We cannot exclude that CFU-f migration is a non-specific effect of phenylhydrazine treatment. However, in view of the literature quoted above, migration of fibroblastoid stromal cells via the blood may represent a general phenomenon which serves the expansion of the haemopoietic stroma in stress-induced haemopoiesis.

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